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13. ABSTRACT (Maximum 200 Words)

Steroid hormones, estrogen, and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptor. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) and E2 ubiquitin-conjugating enzyme, UbcH7 as coactivators of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP and UbcH7 may contribute to the development of breast cancer. We have examined this possibility by studying the expression patterns of E6-AP, UbcH7 and estrogen receptor- alpha (ER) in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. To study the expression profile of E6-AP and UbcH7 in human breast tumors, we examined 100 different human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, our data also demonstrate that ~80% human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. Furthermore, we found that E6-AP modulates the expression levels of ER both in vitro and in vivo. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins E6-AP and UbcH7.

14. SUBJECT TERMS
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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (Nicholson 1979; Nicholson et al. 1986; Horwitz 1994; Nicholson et al. 1995; Parker et al. 1997; Morris et al. 2001). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (Benner et al. 1988; Clarke et al. 1989; Clarke et al. 1992; Elledge et al. 2000). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, the estrogen (ER) and progesterone (PR) receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (Horwitz et al. 1996; McKenna et al. 1998; McKenna 1999; Chen 2000). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (Bocquel et al. 1989; Meyer et al. 1989; Shemshedini et al. 1992). A number of coactivators have been cloned to date, including SRC-1 (Onate et al. 1995), TIF2 (GRIP1) (Hong et al. 1996; Voegel et al. 1996; Hong et al. 1997; Voegel et al. 1998), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (Anzick et al. 1997; Chen et al. 1997; Li et al. 1997; Takeshita et al. 1997; Torchia et al. 1997), PGCs (Puigserver et al. 1998), SRA (Lanz et al. 1999), CBP (Ikonen et al. 1997; Aarnisalo et al. 1998; Fronsdal et al. 1998), **E6-associated protein (E6-AP), and ubiquitin conjugating enzymes such as UbcH5B, UbcH7 and Ubc9** (Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000) and this list is growing rapidly day by day.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (Pugh and Tjian 1992; Tjian and Maniatis 1994). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone acetyl transferase, HAT, activity (Ogryzko et al. 1996; Anzick et al. 1997; Li et al. 1997; Spencer et al. 1997; McKenna et al. 1998; Collingwood et al. 1999; Chen 2000) and members of SWI/SNF complex contain an ATPase activity (Dunaief et al. 1994; Muchardt et al. 1996; Wang et al. 1996; Reyes et al. 1997). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic interactions between histones and their substrate DNA (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Yang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (Xu et al. 1998; McKenna 1999; Leo and Chen 2000; Xu et al. 2000). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and Ubc9 and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (Imhof and McDonnell 1996; McKenna et al. 1998; Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (Fraser et al. 1997; Makino et al. 1997; Masuyama and MacDonald 1998). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (Nawaz et al. 1999b). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically

interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (Huibregtse et al. 1991; Huibregtse et al. 1993). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (Scheffner et al. 1993; Huibregtse et al. 1995b; Huibregtse et al. 1995a). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and many E2 ubiquitin conjugating enzymes (UBCs). The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (Ciechanover 1994; Ciechanover and Schwartz 1994).

The E2 ubiquitin conjugating enzymes of the ubiquitin pathway, UbcH5B and UbcH7 (UBCs) also act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner. Treatment of mammalian cells with the proteasome inhibitor, MG132, which specifically blocks the protease activity of the proteasome, blocks ER degradation. This suggests that ER protein is degraded through the ubiquitin-proteasome pathway (Nawaz et al. 1999a). In addition, our results also suggest that the estrogen-dependent degradation of ER correlates with hormone-dependent ER activation because MG132 not only blocks ER protein degradation but also block its activation. Our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UBCs, UbcH5B and UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). These observations raise the question as to why ubiquitin pathway enzymes and ubiquitin-dependent protein degradation are linked to steroid hormone receptor activation. Considering that the transcriptionally active receptor is associated with a diverse group of proteins and forms a preinitiation complex, it is possible that subsequent to receptor activation of transcription, ubiquitin mediated degradation of the receptor may be a mechanism which dissociates the preinitiation complex. It could be necessary to dissociate the preinitiation complex through targeted protein degradation since the synergistic interactions of multiple transcription factors may make passive dissociation of hormone and coactivators impossible or time consuming. Additionally, it is possible that hormone-induced receptor degradation serves to control physiological responses to steroid hormones ultimately limiting the expression of steroid-responsive genes.

It has been shown that altered expression of one nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (Anzick et al. 1997). Recenly, it has also been shown that another steroid receptor coactivator, SRA, is also elevated in breast tumors (Murphy et al. 2000). Furthermore, we have also shown that E6-AP is

overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (Sivaraman et al. 2000).

The purpose of this research is to explore the possibility that the altered expression of UbcH5B, UbcH7 and E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this possibility by studying the expression profile of UbcH5B, UbcH7, and ER in various breast cancer cell lines and breast tumor biopsy samples. We also proposed to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, UbcH5B and UbcH7. We have examined the expression patterns of E6-AP, UbcH7, with that of ER in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have also correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. Unfortunately, due to the lack of good UbcH5B antibody, we are unable to study the expression profile of UbcH5B. Instead of UbcH5B, we have examined the expression profile of E6-AP in several breast cancer cell lines and breast biopsy tumors. In order to study the expression profile of E6-AP and UbcH7 in human breast tumors, we have examined 100 different breast cancer biopsy samples for the expression profile of E6-AP, UbcH7 and ER. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Additionally, we have also examined the expression profile of E6-AP, UbcH7 and ER in early and intermediate stage tumors. We found that there is no correlation between the expression profile of E6-AP and ER and UbcH7 and ER in early and intermediate stage tumors. The expression of E6-AP is down in 80% of invasive breast cancers and expression of E6-AP is inversely correlated with ER in invasive breast cancers. Furthermore, we found that E6-AP modulates the expression levels of ER both in vitro and in vivo. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, E6-AP and UbcH7. In order to achieve this goal, we have constructed the expression vectors for stable cell lines. Our data suggest that these vectors produce biologically functional coactivator proteins, E6-AP and UbcH7. However, we failed to overexpress either E6-AP or UbcH7 in stable cell lines both by constitutive expression system and inducible expression system.

Body

In this original proposal, we hypothesized that ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and an E3 ubiquitin-protein ligase, E6-AP, are important modulators of the steroid hormone receptor-mediated signal transduction pathway, cell growth, and cell cycle control in the context of breast cancer development. In order to test this hypothesis we propose following objectives:

- **Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.**
- **Design and development of stable in vitro models of UbcH5B and UbcH7 overexpression in the breast cancer cell lines.**

- **Analysis of the growth properties of stably transfected cell lines that overexpress UbcH5B and UbcH7 and in vivo analysis of tumorigenicity of these stably transfected cell lines in athymic nude mice.**

Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.

One of the aims of this proposal is to test the expression of endogenous UbcH5B, UbcH7 and ER in human breast cancer cell lines and human breast tumor biopsies. Then compare the expression profile of UbcH5B and UbcH7 with that of ER. We have examined expression levels of UbcH7, E6-AP, and ER in 100 different breast tumors and expression of p53 in 20 different tumors. Additionally, we have also examined the expression profile of UbcH7, E6-AP and ER in different breast cancer cell lines. Due to the lack of the availability of the UbcH5B antibody, we are unable to examine the expression profile of UbcH5B. Furthermore, we were not successful in generating a good UbcH5B antibody. Since these proteins are also targets of the ubiquitin-proteasome pathway, we did not analyze the mRNA levels of UbcH5B. Additionally, UbcH5B and UbcH7 both act as E2 ubiquitin-conjugating enzymes for E6-AP, therefore, we also examined the expression of E6-AP in different breast cancer cell lines and breast tumor biopsies. We found an inverse correlation between the expression of E6-AP and the expression of ER in human biopsy tumor samples, and we did not find any statistically significant correlation between the expression profile of UbcH7 and ER.

<u>**Task 1.** Expression analysis of UbcH7, ER and E6-AP in different breast cancer cell lines.</u>

<u>**A.** UbcH7 expression</u>

We have analyzed the expression profile of UbcH7, ER and E6-AP in different breast cancer cell lines such as MCF-7, T47-D, ZR75-1 and MDA-MB-231. As a control we have also examined the expression profile of UbcH7, ER and E6-AP in HeLa (a cervical carcinoma cell line) cells. As shown in Figure 1, HeLa cells express high levels of UbcH7 protein compare to that of different breast cancer cell lines (MCF-7, T47-D, ZR75-1 and MDA-MB-231). Furthermore, in HeLa cells, the UbcH7 expression is both cytoplasmic and nuclear. The expression level of UbcH7 in MCF-7, T47-D, ZR75-1 and MDA-MB-231 is moderate compare to that of HeLa cells. In T47-D cells the expression of UbcH7 is totally nuclear whereas in other breast cancer cell lines, MCF-7, ZR75-1 and MDA-MB-231, weak cytoplasmic staining of UbcH7 was observed, in addition to nuclear staining.

<u>**B.** ER expression</u>

Since we want to compare the expression profile of UbcH7 with that of ER, we also analyzed the expression of ER-alpha in MCF-7, T47-D, ZR75-1 and MDA-MB-231 cell lines. In this case HeLa cell line was used as a negative control. As shown in Figure 2, HeLa cells are negative for ER expression. Similarly, in the breast cancer cell line, MDA-MB-231, the ER expression was undetectable. However, this cell line expresses UbcH7 at moderate level. In contrast to MDA-MB-231, the MCF-7, T47-D and ZR75-1 lines express both UbcH7 and ER-alpha. The ER expression is nuclear in these cell lines.

C. E6-AP expression

Since UbcH7 act as an E2 ubiquitin-conjugating enzyme for E6-AP and both the UbcH7 and E6-AP act as coactivators for ER, we decided to analyze the expression profile of E6-AP in breast cancer cell lines. As shown in Figure 3, the breast cancer cell lines, MCF-7, T47-D, ZR75-1 and MDA-MB-231 express high levels of E6-AP. The E6-AP expression is both cytoplasmic and nuclear in MCF-7, ZR75-1 and MDA-MB-231 cell lines. The MDA-MB-231 cell line expresses more E6-AP in nucleus than in the cytoplasm. Similar to UbcH7, the expression of E6-AP in T47-D cells is purely nuclear. In this case HeLa cells were used as a positive control for E6-AP expression.

Task 2. Effect of steroids on the expression of UbcH7 and E6-AP.

It is possible that steroid hormones (estrogens/progesterones) may regulate endogenous expression of UbcH7 and E6-AP in breast cancer cell lines. To test this possibility, MCF-7, a hormone-dependent breast cancer cell line was grown in the medium containing stripped serum for a week. Afterward, cells were grown either in the absence or presence of steroid hormones for 48 hours and the expression patterns of UbcH7 and E6-AP were determined by fluorescent immunocytochemistry. As shown in Figure 4, in MCF-7 cells, estrogen treatment has no significant effect on the expression profile of UbcH7. The UbcH7 expression is identical both in the absence and presence of estradiol. Similarly, progesterone treatment also has no significant effect on the expression levels of UbcH7 in MCF-7 cells (data not shown). Next we ask whether steroids treatment has any effect on the expression levels of UbcH7 in T47-D cells. As shown in Figure 5, estrogen treatment has no effect on the expression levels of UbcH7 in T47-D cells. The expression levels of UbcH7 are identical both in hormone treated and untreated cells. The same is true for progesterone (data not shown). These data suggest that the expression of UbcH7 is not under the control of steroid hormones.

Next we ask whether steroids regulate the expression of E6-AP. To test the effect of estrogen on the expression pattern of E6-AP, MCF-7 cells were grown in the medium containing stripped serum for a week. Then, cells were treated with either estrogen or vehice for 48 hours and the expression patterns of E6-AP was determined by fluorescent immunocytochemistry. Figure 6 suggests that the estrogen treatment have no significant effect on the expression of E6-AP. The E6-AP expression levels are identical both in the presence and absence of hormone. This data suggests that E6-AP regulation is not under the control of steroids.

As a control for these experiments, we also analyzed the effect of estrogens on the expression of PR and ER. It has been established that estrogen upregulate the expression of PR protein and it down regulate the levels of ER in MCF-7 cells (Lonard et al. 2000). As expected, Figure 7 demonstrate that estrogen treatment increases the expression of PR protein. In contrast, estrogen down regulates ER expression.

Task 3. Expression analysis of ER-alpha, UbcH7 and E6-AP in breast tumor samples.

As mentioned above, the ubiquitin pathway enzymes, UbcH7 and E6-AP act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which

is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner via the ubiquitin-proteasome pathway. Additionally, our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). To explore the possibility that the altered expression of UbcH7 and E6-AP may contribute to the development of breast cancer, we analyzed the expression profile of UbcH7, E6-AP and ER in 100 advanced stage breast tumor biopsy samples by Western blot analysis and immunohistochemistry. Figure 8 and 12 show the expression profile of ER-alpha in 56 different human tumor samples. 23 (41%) out of 56 breast tumor samples express significant amount of ER-alpha, however, 33 (59%) tumors express no or degraded forms of ER protein. Since we want to correlate the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies, we examined the expression profile of UbcH7 and E6-AP in these tumors. Figure 9 and 12 show the expression profile of UbcH7 in human breast tumor samples. As shown in Figure 9, majority of the tumors expresses UbcH7. Only 21(%) tumors are negative for UbcH7. Furthermore, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples.

Since UbcH7 acts as an E2 ubiquitin-conjugating enzyme for E6-AP, we also examined the expression profile of E6-AP in these human breast tumoe samples. To study the expression profile of E6-AP in human breast tumors we performed Western blot analysis using an E6-AP specific antibody. Figure 10 shows the expression of E6-AP in 56 different tumor samples. The majority (82%) of the tumors expresses E6-AP. Furthermore, we found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant (Figure 12).

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. In the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Figure 11, p53 expression was not detectable in most tumors except tumor number 7, 10, 13 and 15. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53. Presently, we are analyzing more tumor samples for p53 expression.

Since we found an inverse correlation between the expression profile of ER-alpha and E6-AP, we analyzed whether E6-AP and ER expression colocalized in breast cancer cell lines. In order to study the colocalization of ER and E6-AP, we performed fluorescent immunocytochemistry on T47-D cells. As shown Figure 13, the expression of E6-AP colocalized with that of ER (see merge figure). These data suggest that it is possible that in vivo E6-AP may promote the degradation of ER through the ubiquitin-proteasome pathway.

We have also examined the expression profile of E6-AP in normal human mammary tissues by immunohistochemistry using anti-E6-AP antibody. Fig. 14 suggests that E6-AP is highly expressed in normal human mammary ducts and almost every epithelial cell express E6-AP protein (Fig. 14).

In order to study the expression profile of E6-AP in human breast tumor samples, we examined the expression profile of E6-AP in different human breast tumor samples and adjacent normal tissues by immunohistochemistry. As mentioned above, E6-AP expression is very high in normal mammary ducts (Fig. 15). However, 80% of human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues (Fig. 15).

Next, we analyze the expression level of E6-AP between different stages of breast cancer by immunohistochemistry and then compare the expression profile of E6-AP within different stages by Wicoxonrank-sum test. This analysis suggests that E6-AP expression is decreased in stage IIB breast tumors (Fig. 16).

Since, the ubiquitin-proteasome pathway is responsible for down regulation of ER, furthermore, we found an inverse correlation between the expression levels of E6-AP and the expression levels of ER in human breast tumors. Based on these observations we hypothesized that it possible that E6-AP modulates the expression levels of ER in the mammary gland by promoting their degradation via the ubiquitin-proteasome pathway. To test this hypothesis, we performed in vitro protein degradation and ubiquitination assay on ER protein both in the absence and presence of E6-AP. ^{35}S-labeled ER protein was synthesized in vitro using TNT-coupled wheat germ extracts. The ^{35}S-labeled ER protein was then incubated with ATP and ubiquitin either in the presence or absence of bacterially expressed E6-AP protein. As shown in Fig. 17, no ER degradation was observed in the absence of E6-AP. The ER degradation was observed only in the presence of E6-AP protein. Furthermore, the proteasome inhibitor, lactacystin was able to block ER degradation.

To further confirmed that E6-AP modules the levels of ER, we also examined the ER protein levels in E6-AP knockout mice. E6-AP knockout mice exhibited an increase in the protein levels of ER in the mammary epithelial cells (Fig. 18). These data confirmed that E6-AP modulates the levels of ER by promoting its degradation through the ubiquitin-proteasome pathway.

Design and development of stable in vitro models of UbcH7 and E6-AP overexpression in the breast cancer cell lines.

<u>**Task 4.** Generation of the expression plasmids for overexpression of UbcH7 and E6-AP.</u>

To construct the expression plasmids for the overexpression of UbcH7 and E6-AP, cDNAs of UbcH7 and E6-AP were are cloned into the mammalian expression vector pcDNA3.1. These vectors produce his-tagged UbcH7 and E6-AP proteins. In order to confirm the functional activity of the his-tagged UbcH7 and E6-AP, we performed the transient transfection assays in HeLa cells. As shown in Figure 19, that the his-tagged UbcH7 and E6-AP were able to enhance the transactivation functions of PR, suggesting that the his-tagged UbcH7 and E6-AP proteins are biologically functional. Presently, we are in the process of generating the stable cell lines that overexpress UbcH7 and E6-AP proteins.

<u>**Task 5.** Development of stable cell lines.</u>

In order to generate stable cell lines that overexpress either UbcH7 or E6-AP, we utilized both constitutive expression system and Tet Off inducible system. We were successful in generating the stable cell lines.

Task 6. Characterization of stable cell lines.

After establishing stable cell lines, we characterized stable lines for the overexpression of either UbcH7 or E6-AP. Our expression analysis data suggest that these stable lines fail to overexpress E6-AP and UbcH7 protein. This suggests that overexpression of UbcH7 and E6-AP proteins are toxic for cells. Since, UbcH7 and E6-AP are the ubiquitin-proteasome pathway enzymes, it is possible that overexpression of UbcH7 and E6-AP may result in degradation of proteins that are required for cell growth and survival.

Statement of work accomplished/in progress

Task 1. Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines. **Accomplished.**
Task 2. Effect of steroids on the expression of UbcH7 and E6-AP. **Accomplished.**
Task 3. Expression analysis of ER-alpha, UbcH7, and E6-AP in breast tumor samples. **Accomplished.**
Task 4. Generation of the expression plasmids for overexpression of UbcH7 and E6-AP. **Accomplished.**
Task 5. Development of stable cell lines. **Accomplished.**
Task 6. Characterization of stable cell lines. **Accomplished.**

Key Research Accomplishments

- Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines has been completed.
- Effect of steroids on the expression of UbcH7 and E6-AP has been studied.
- The expression analysis of ER, UbcH7, and E6-AP has been analyzed.
- Expression profile of E6-AP has been compared with that of ER expression.
- Expression profile of UbcH7 has been compared with that of ER expression.
- It has been established that E6-AP promote ER degradation both in vitro and in vivo.
- Generation of the expression plasmids for overexpression of UbcH7 and E6-AP has been completed.
- The biological activities of His-tagged UbcH7 and E6-AP has been analyzed.
- Stable cell lines have been established for UbcH7 and E6-AP.
- It has been established that overexpression of UbcH7 and E6-AP is toxic for cells.

Reportable Outcomes

1. X. Gao, S. K. Mohsin, Z. Gatalica, F. Yan, and Z. Nawaz. 2004. E6-associated protein is involved in the carcinogenesis of human breast and prostate. (In Preparation). (see appendix 2).

Conclusions

We have successfully analyzed the expression of UbcH7, E6-AP and ER in different breast cancer cell lines. Additionally, we have also examined the effects of steroids on the expression profile of UbcH7, E6-AP and ER. In order to study the expression profile of UbcH7, E6-AP and ER in human breast tumors, we have examined 100 different human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. Furthermore, we show that E6-AP promotes ER degradation both in vitro and in vivo. However, we did not observe any correlation between the expression of UbcH7 and ER. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. We have generated stable cell lines for UbcH7 and E6-AP. However, overexpression of UbcH7 and E6-AP proteins are toxic for cells.

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Appendices

1. Figures 1-19

2. Manuscript

Appendix 1

Figures 1-19

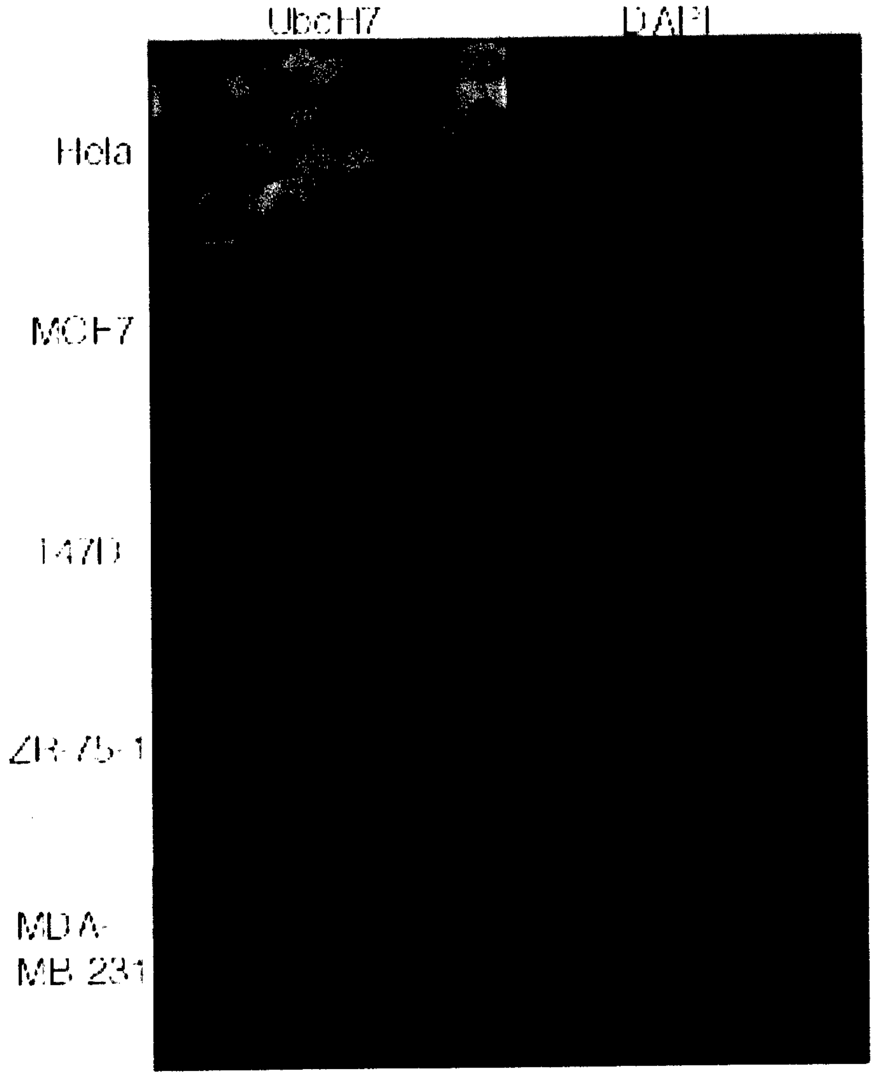

Figure 1: Expression analysis of UbcH7 in different cell lines (Hela, MCF7, T47DZR-75-1 and MDA-MB-231). Cells were grown on a chamber slide for 24 hrs and UbcH7 expression was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

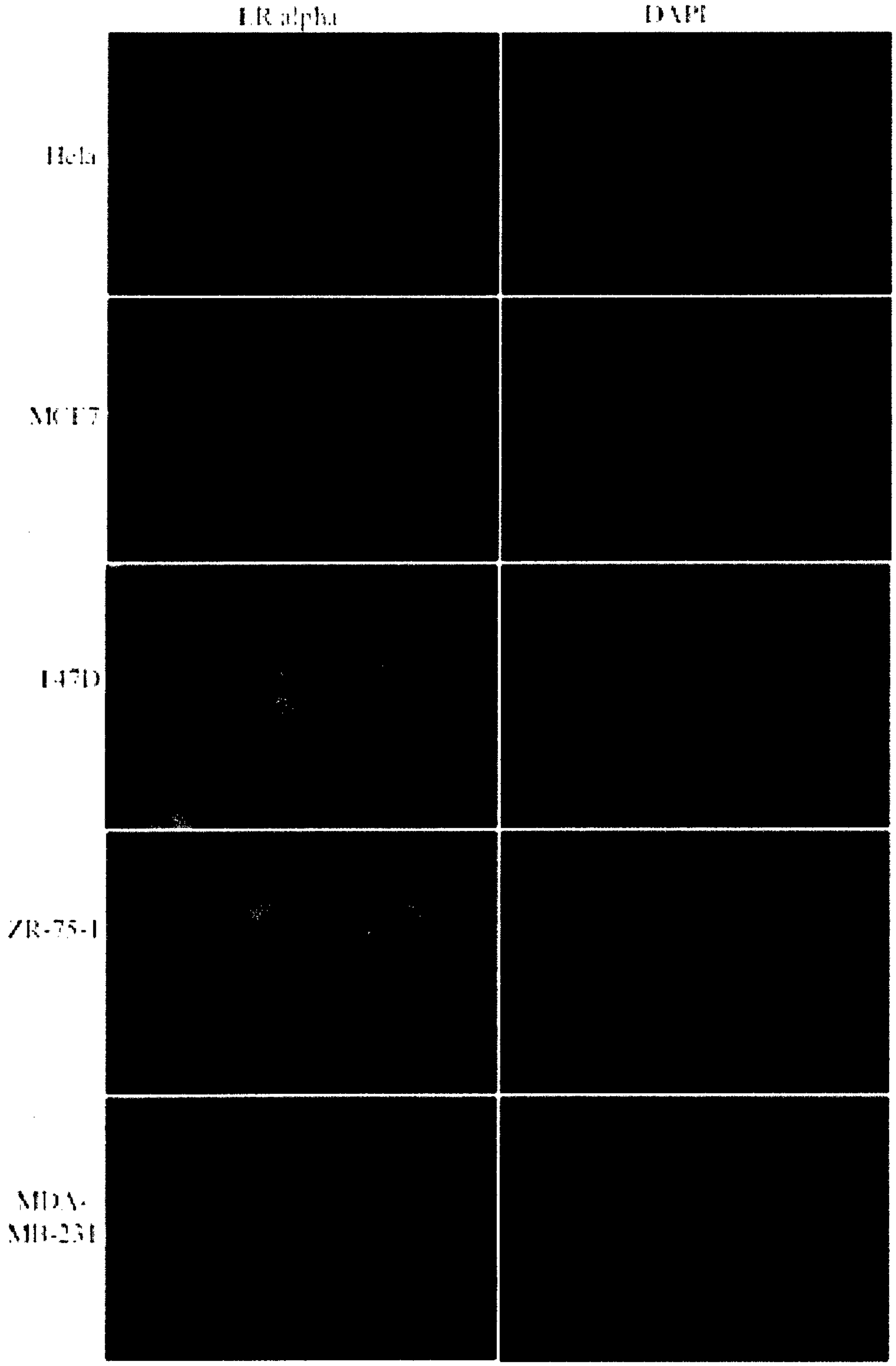

Figure 2: Expression analysis of ER-alpha in different cell lines (Hela, MCF7, T47D, ZR-75-1and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and ER-alpha expression was analyzed by fluorescent immunocytochemistryusing an anti-ER-alpha antibody. Positive signal for ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. ER-alpha. ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

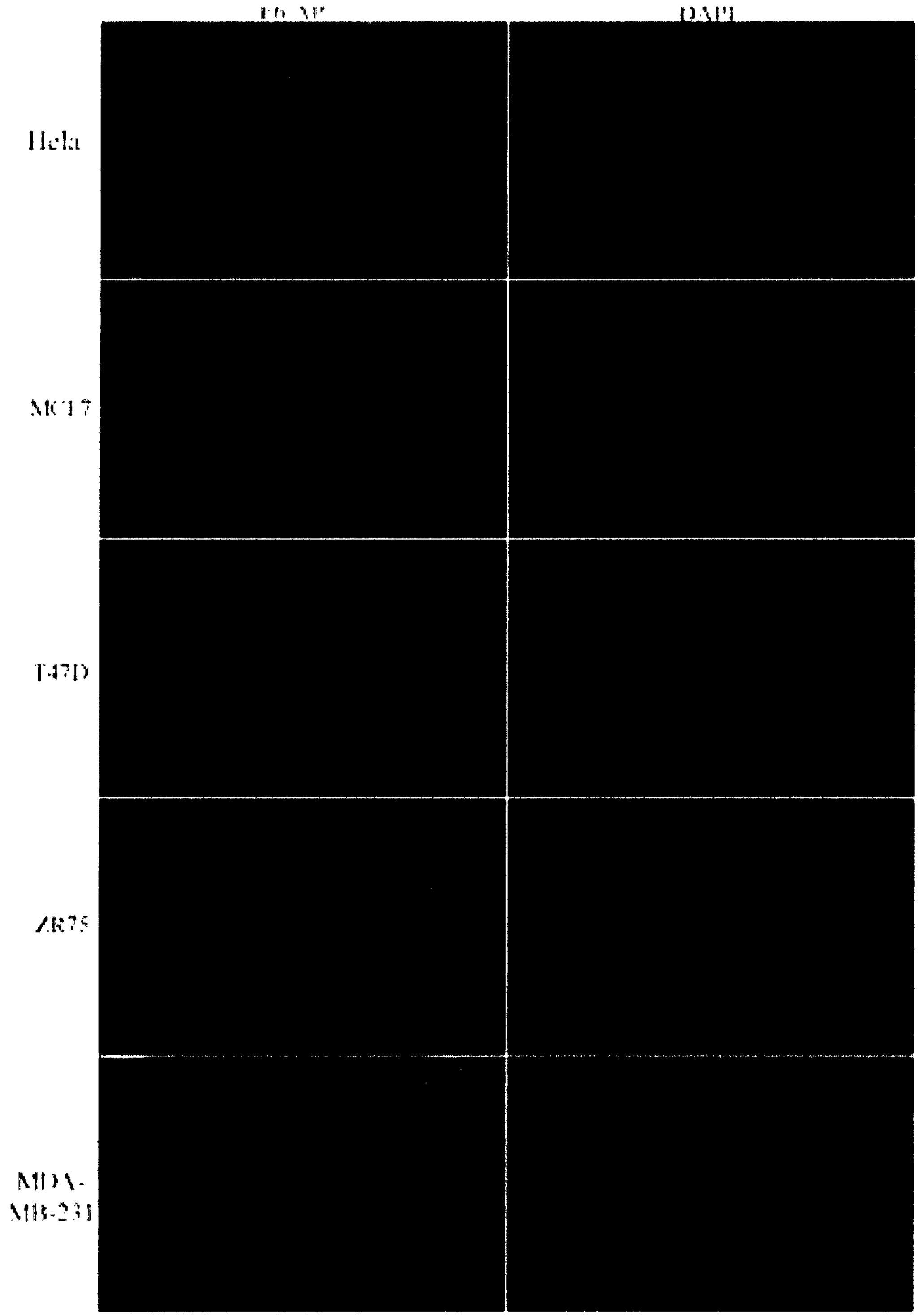

Figure 3: Expression analysis of E6-AP in different cell lines (Hela, MCF7, T47D, ZR-75-1 and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and E6-AP expression was analyzed by fluorescent immunocytochemistryusing an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAPI staining. E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

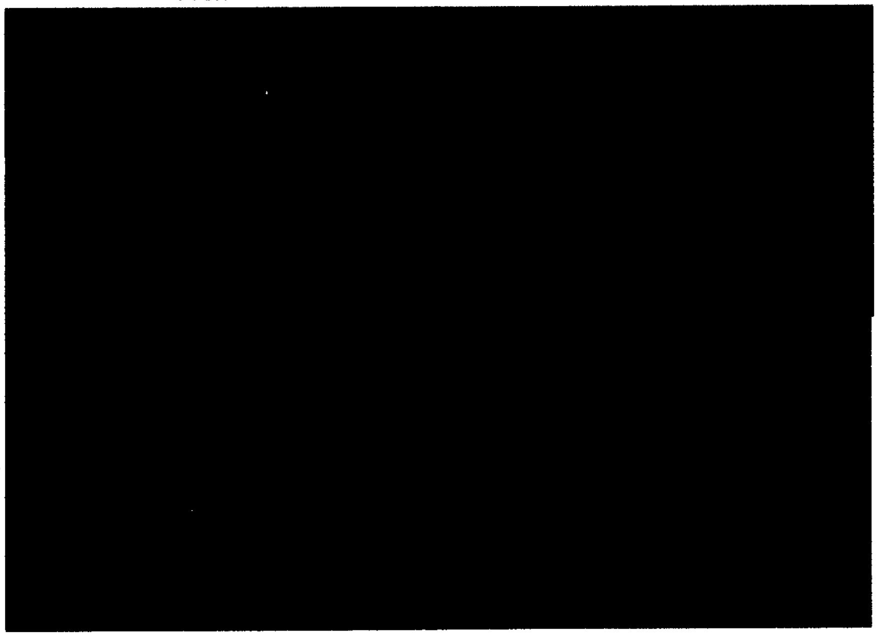

Figure 4: Effect of estrogen on the expression of UbcH7 in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAPI staining. UbcH7, UbcH7 expression profile; DAPI, DAPI staining for nucleus.

UbcH7 DAPI

-H

+E

Figure 5: Effect of estrogen on the expression of UbcH7 in T47D cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAPI staining. UbcH7, UbcH7 expression profile; DAPI, DAPI staining for nucleus.

E6-AP DAPI

-H

+E

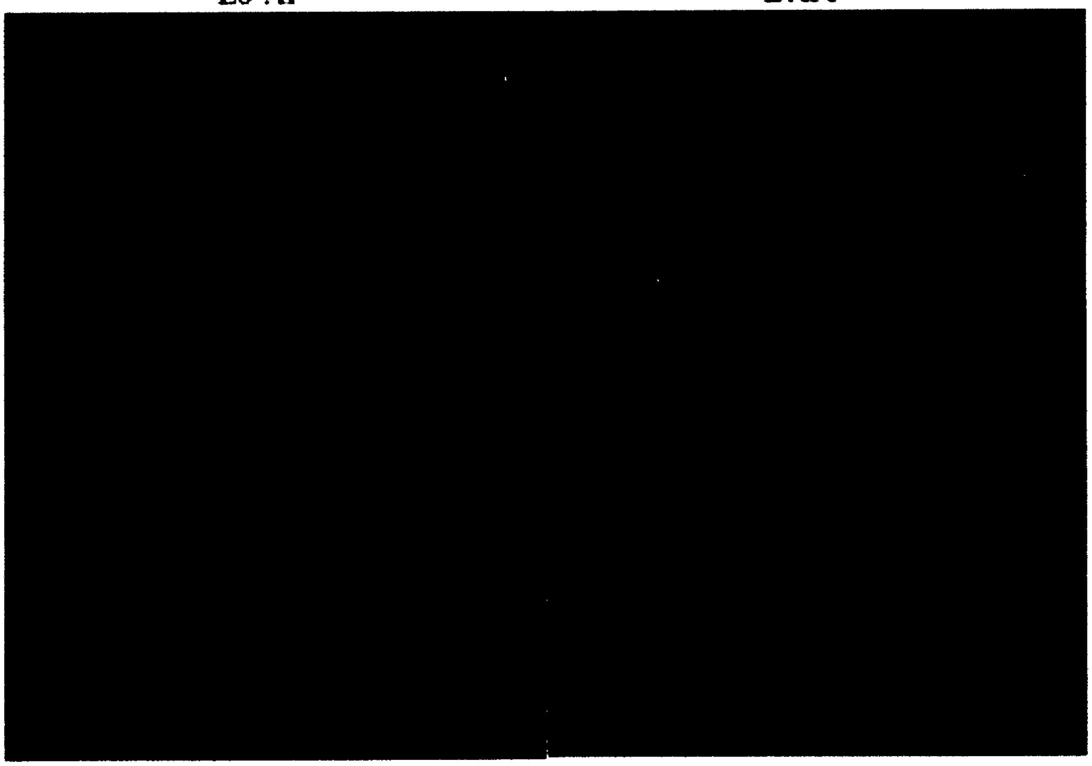

Figure 6: Effect of estrogen on the expression of E6-AP in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous E6-AP was analyzed by fluorescent immunocytochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAPI staining. E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

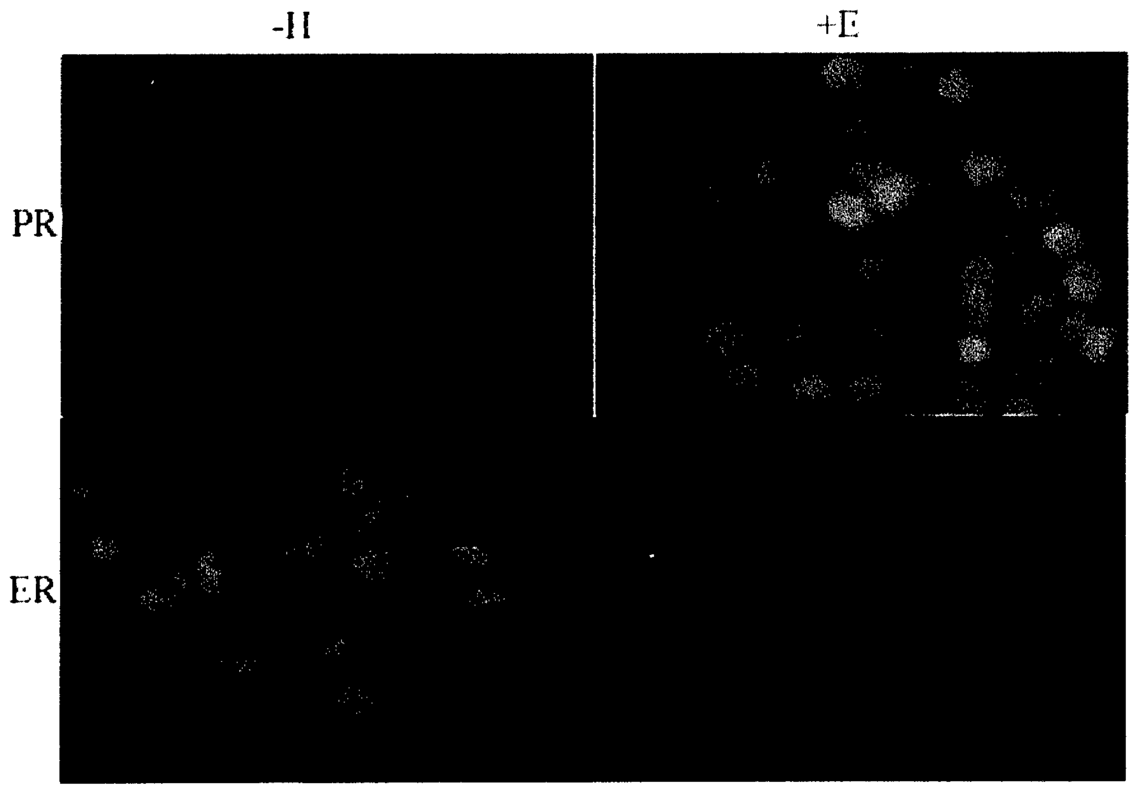

Figure 7**:** Effect of estrogen on the expression of PR and ER-alpha in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous PR and ER-alpha was analyzed by fluorescent immunocytochemistry using anti-PR and anti-ER-alpha antibodies. Positive signal for PR and ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. PR, PR expression profile; ER, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

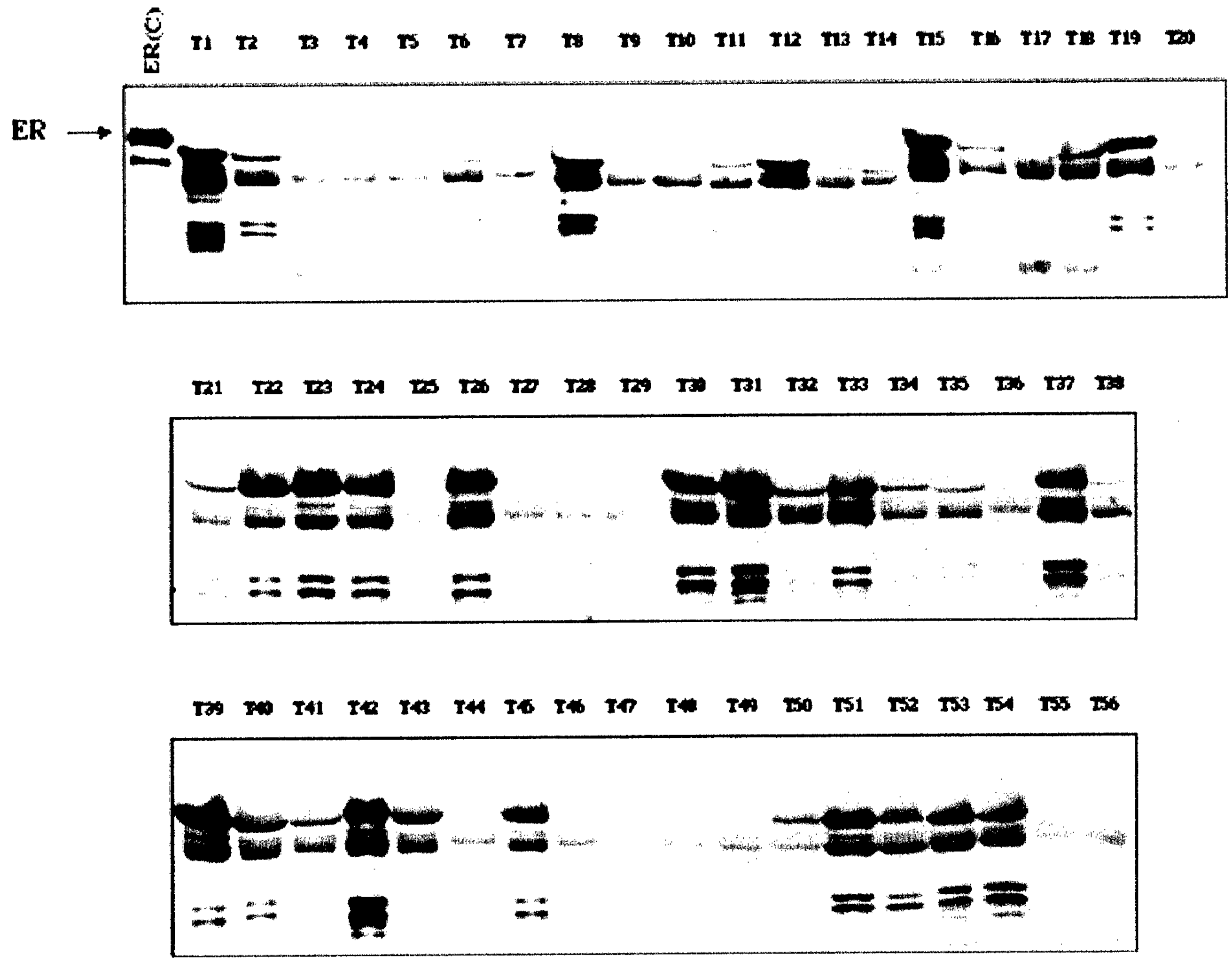

Figure 8: Expression analysis of ER-alpha in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of ER-alpha was analyzed by Western blot analysis using an anti-ER antibody. C, Purified ER protein was used as a control. T1-T56 represent different tumor samples.

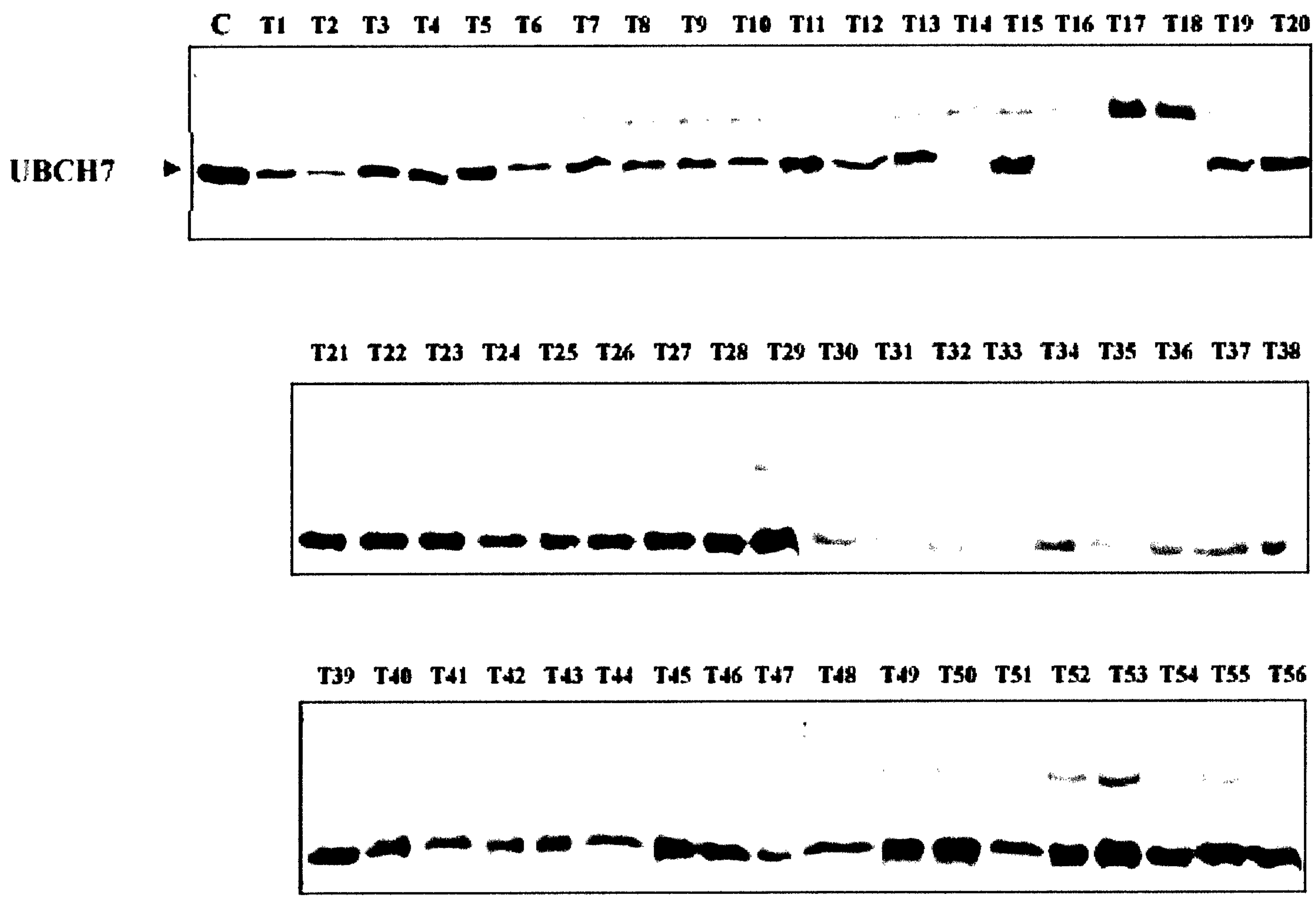

Figure 9: Expression analysis of UbcH7 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of UbcH7 was analyzed by Western blot analysis using an anti-UbcH7 antibody. C, Purified UbcH7 protein was used as a control. T1-T56 represent different tumor samples.

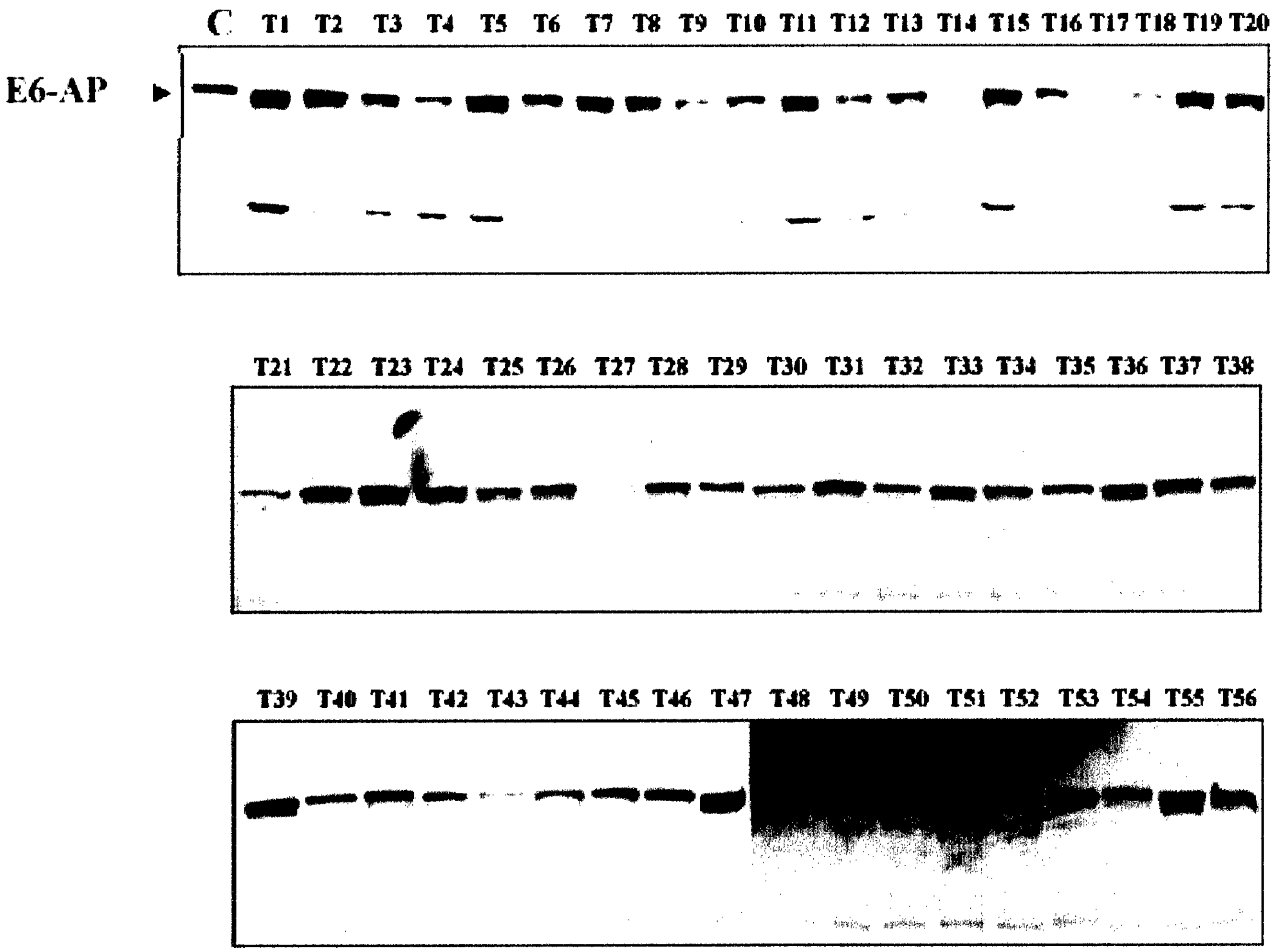

Figure 10: Expression analysis of E6-AP in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of E6-AP was analyzed by Western blot analysis using an anti-E6-APantibody. C, Purified E6-AP protein was used as a control. T1-T56 represent different tumor samples.

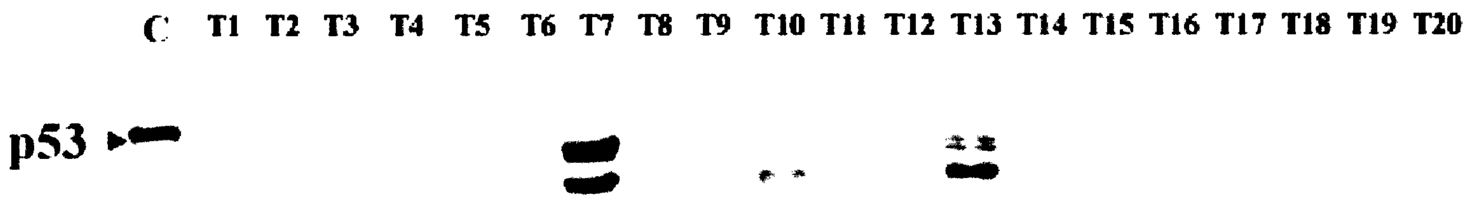

Figure 11: Expression analysis of p53 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of p53 was analyzed by Western blot analysis using an anti-p53 antibody. C, Purified p53 protein was used as a control. T1-T20 represent different tumor samples.

Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7
1	++++	++++	++	21	+	+	+++	41	++	+	+
2	+++	+	+	22	+++	++	+++	42	++	++++	+
3	++	-	+++	23	++++	++++	+++	43	+	++	+
4	+	-	+++	24	+++	+++	++	44	+	++	+++
5	++++	-/+	++++	25	++	-	+++	45	++	++	+++
6	++	-/+	+	26	++	++++	++	46	+++	-/+	+++
7	++	-	++	27	-/+	-/+	+++	47	+++	-/+	++
8	+++	+++	++	28	++	-/+	++++	48	+	-/+	++
9	+	-/+	++	29	++	-/+	++++	49	++	-/+	+++
10	++	-/+	++	30	++	+++	-/+	50	++++	+	+++
11	+++	+	++++	31	++++	++++	-/+	51	++++	+++	++
12	++	++	+++	32	++	+	-/+	52	++++	+	++++
13	++	-/+	+++	33	+++	++	-/+	53	+++	++	++++
14	-/+	-/+	-/+	34	+++	+	-/+	54	+++	++	+++
15	++++	++++	+++	35	++	+	-/+	55	++++	-/+	+++
16	++	-/+	-/+	36	+++	-	-/+	56	+++	-/+	+++
17	-/+	-	-/+	37	+++	++++	-/+				
18	+	+	-/+	38	+++	+	-/+				
19	++++	++	++	39	++++	+++	++				
20	++++	-	++++	40	+	++	+				

Figure 12: Correlation of the expression of E6-AP and UbcH7 with that of ER-alpha in breast tumors. Expression levels of E6-AP, ER-alpha and UbcH7 from Western blot analysis was artificially graded according to the density of the bands. "-" represents negative expression, whereas "-/+" represents very low expression. From "+" to "++++" represent the gradually increasing levels of expression from low to high. Sprearman Rank Correlation Coefficient for the expression of E6-AP with that of ER-alpha is 0.38, p=0.004. However, there is no correlation between UbcH7 and ER-alpha expression and UbcH7 and E6-AP expression.

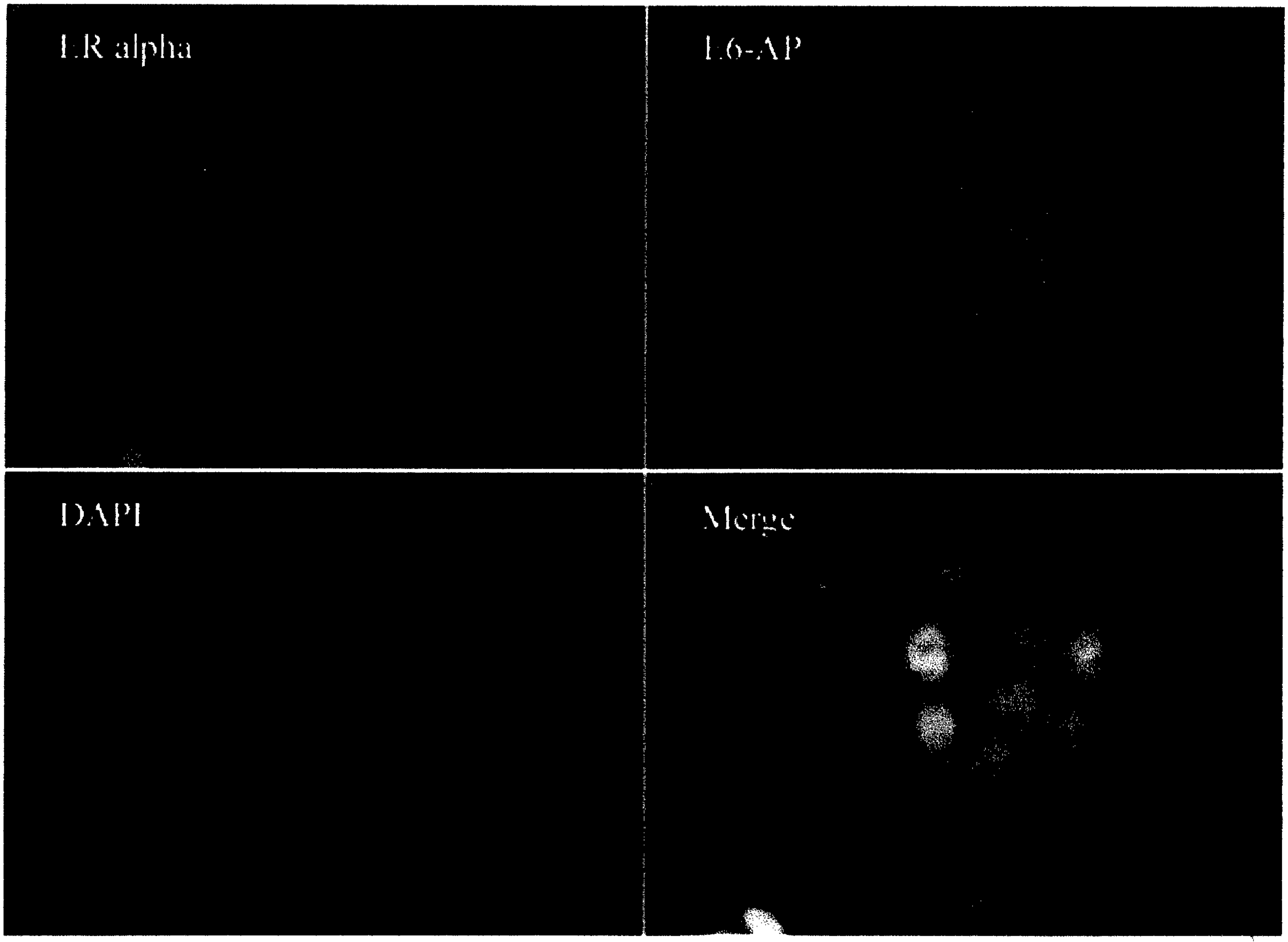

Figure 13: Colocalization of E6-AP and ER-alpha in T47D human breast cancer cell line by immunocytochemistry. Cells were grown on a chamber slide for 24 hours and the expression of endogenous E6-AP and ER-alpha was analyzed by fluorescent immunocytochemistry using either an anti-E6-AP or ER-alpha antibody. Positive signal for E6-AP is seen as (red) spots, ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. Yellow spots (merge) indicate colocalization of ER-alpha and E6-AP. ER-alpha, ER-alpha expression profile; E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

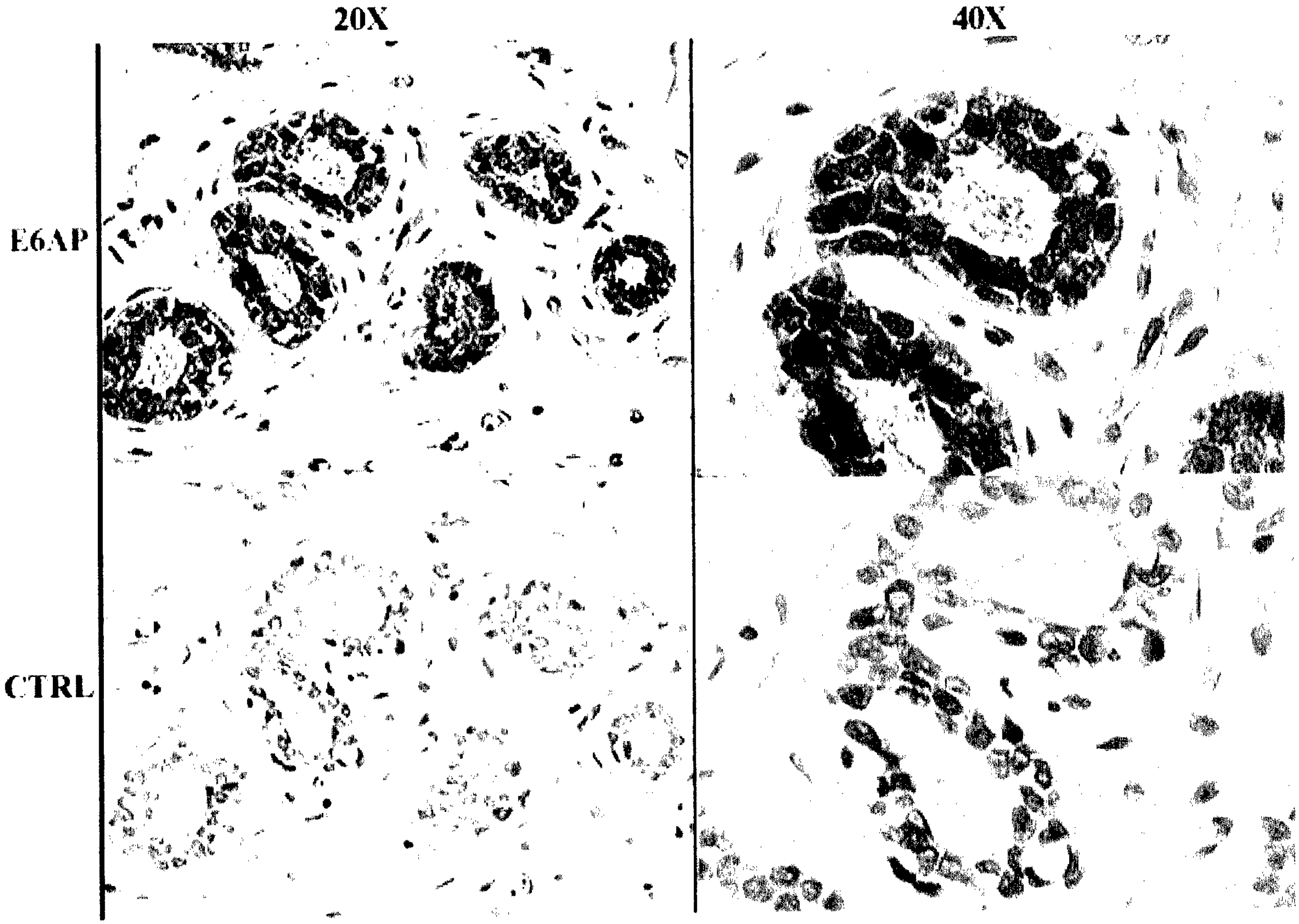

Figure 14: Expression analysis of E6-AP in normal human breast tissues by immunohistochemistry. The expression of endogenous E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. E6-AP, E6-AP polyclonal antibody; CTRL, no primary antibody.

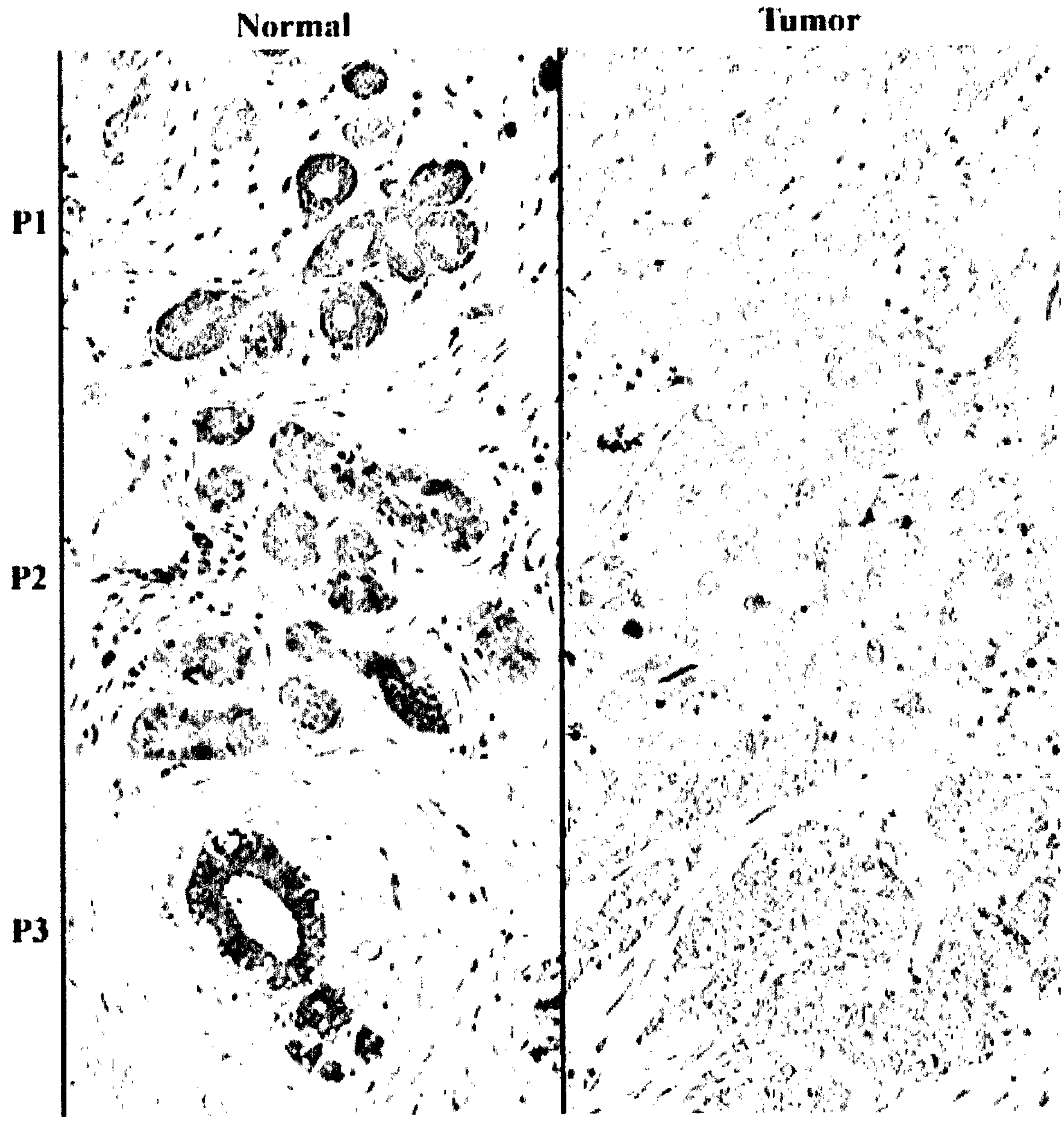

Figure 15: Expression analysis of E6-AP in human breast tumors and adjacent normal tissues by immunohistochemistry. The expression of E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. P1, Patient1; P2, Patient 2; P3, Patient 3.

Comparison of the Expression Level of E6-AP between Different Stages of Breast Tumors by Wilcoxon Rank-sum Test

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
I & IIB	32	7, 2	<0.01*
IIA & IIB	52.5	7, 7	>0.05
IIIA & IIB	59.5	6, 1	<0.01*
I & IIA	115	9, 5	>0.1
I & IIIA	50.5	6, 3	>0.1

Figure 16. Statistical analysis of the expression levels between different stages of breast tumors. Based on the distribution of E6-AP in different stages of breast tumors (shown in Figure 17), the differences of the levels of E6-AP expression between different stages of breast tumors were analyzed by Wilcoxon rank-sum test. Since Figure 17 suggested that the expression of E6-AP decreased gradually from stage I to stage IIB, and then it goes up again in stage IIIA, we compared the differences of expression between stage IIB with that of every other stage. The expression of E6-AP in stage I, stage IIIA were significantly different from that of stage IIB, suggesting that stage IIB is the lowest point in E6-AP expression.

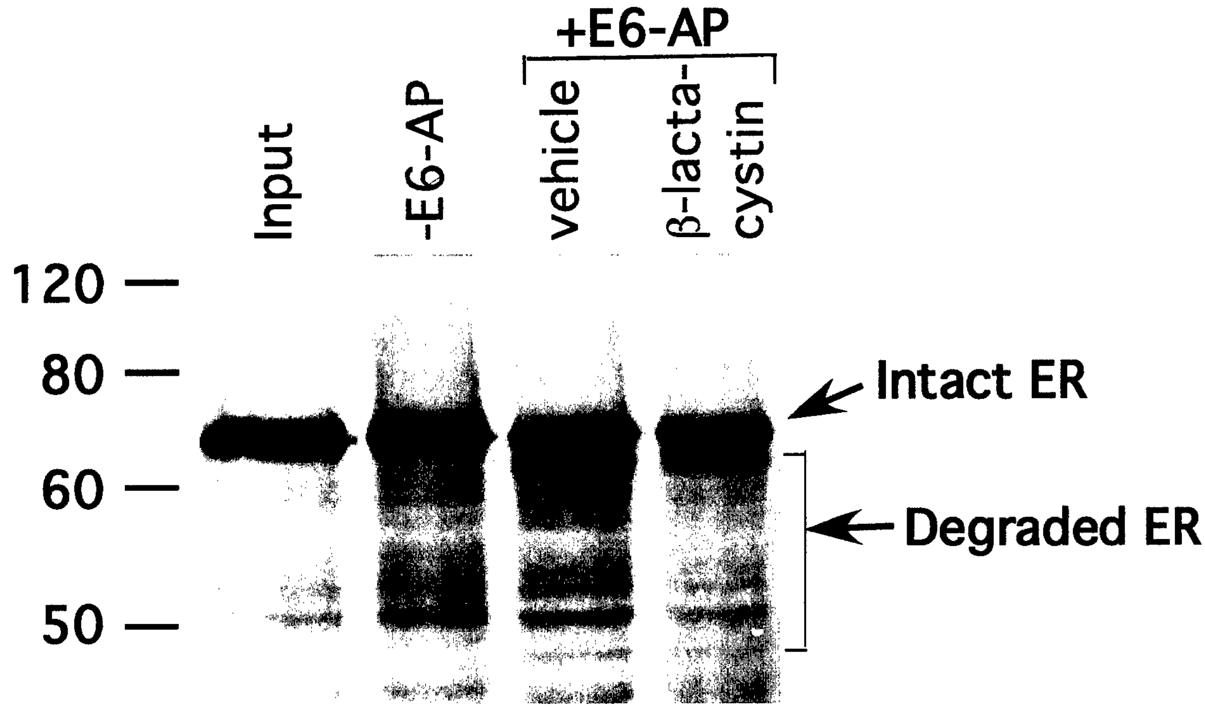

Fig. 17: E6-AP promotes ER degradation via the ubiquitin-proteasome pathway. Radiolabeled human ER was synthesized using TNT-coupled wheat germ extracts. Then ER was incubated with ubiquitin and ATP either in the absence of E6-AP (-E6-AP) or in the presence of E6-AP (+E6-AP). Vehicle lane represents the reaction incubated with E6-AP in the absence of proteasome inhibitor. Lactacystin lane represents the reaction incubated with E6-AP and proteasome inhibitor. Input lane represents the reaction that is not incubated with ubiquitin and ATP.

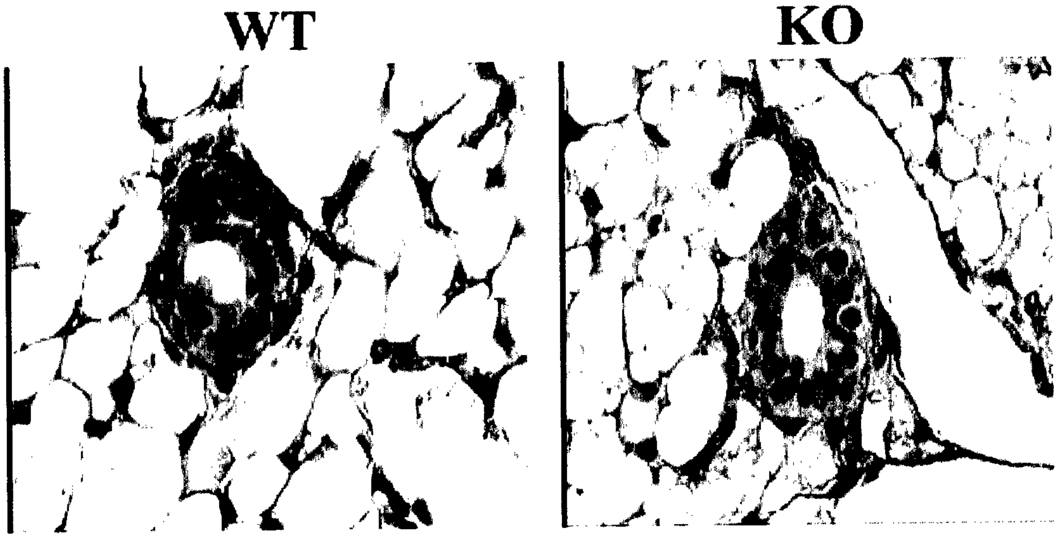

Fig. 18: Expression analysis of ER in mouse mammary glands. ER expression in mouse mammary glands was analyzed by immunohistochemistry using an anti-ER antibody. ER expression in wild-type (WT) and E6-AP knockout (KO) glands. Positive signal is seen as dark (brown) spots.

Relative Expression of E6-AP in Human Mammary Tumors and Their Adjacent Normal Tissues

		NORMAL TISSUES		Total
		High	Low	
TUMOR	High	2	0	2
	Low	8	2	10
	Total	10	2	12

*Chi-Square Test, $p<0.025$

Figure 19. The distribution of different levels of E6-AP expression in normal and malignant human breast tissues. Comparing the expression level of E6-AP in each pair of samples,there are four kinds of outcomes: equally high in both tumor and normal tissues; equally low in both tumor and normal tissues; high in tumor and low in normal tissues; low in tumor and high in normal tissues. It is noticed that the majority of the samples (8 out of 12) express lower levels of E6-AP in tumors in comparison to their adjacent normal tissues. Chi-Square test shows that the differences between the four groups are statistically significant ($p<0.025$).

Appendix 2

Manuscript

E6-associated protein, E6-AP is involved in the carcinogenesis of human breast and prostate

Xiuhua Gao, Syed K. Mohsin, Zoran Gatalica, Feng Yan and Zafar Nawaz[2]

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Abstract

The E6-associated protein, E6-AP, is a dual function protein. It acts as an E3 ubiquitin-protein ligase as well as a steroid receptors coactivator. Considering the importance of steroid receptors and their coactivators in the normal development and tumorigenesis of reproductive organs of both genders, we studied the roles of E6-AP in the tumorigenesis of female breast and male prostate tissues. We found that the expression of E6-AP protein is decreased in human invasive breast carcinomas and prostate carcinomas compared to their adjacent normal tissues, and this downregulation of E6-AP is accompanied by the upregulation of estrogen receptor-α/androgen receptor, in breast/prostate carcinomas. Furthermore, in vitro studies and studies of E6-AP knockout animals indicated that E6-AP regulates the expression and function of ERα by improving its degradation through the ubiquitin-proteasome pathway.

Introduction

Breast and prostate carcinomas are among the major causes of cancer-related deaths in American women and men, respectively. It is well established that the steroid hormones,

estrogen, progesterone and androgen are the principal regulators in mammary gland and/or prostate gland development and function (1, 2). Functioning through their cognate intracellular receptors, estrogen receptors (ER), progesterone receptors (PR) and androgen receptor (AR), which all belong to the nuclear receptor superfamily and act as transcription factors, these steroids not only regulate the normal growth and development of their target tissues, but also play important roles in the tumorigenesis of these tissues (1, 2). As the mediator of steroid hormone activity, abnormal expression or function of ER and AR has been associated with cancers of breast and prostate, respectively. Besides, the development of resistance to antiestrogen or LHRH (luteinizing hormone-releasing hormone) antigonist therapy for breast and prostate cancers, respectively, is also related to aberrant expression, mutation of the genes and abnormal functioning of the respective steroid receptors (Anderson, 2002; Culig et al., 2002). Currently, the detailed molecular mechanisms by which the steroid receptors and their coactivators regulate the development and progression of breast and prostate cancers remain largely unknown. In this study, we want to explore the roles of the novel steroid receptor coactivator, E6-associated protein (E6-AP) in the development and progression of breast and prostate carcinomas.

E6-AP is an E3 ubiquitin-protein ligase as well as a steroid receptor coactivator (3). Ubiquitin-proteasome pathway is involved in the degradation of steroid receptors, including ER, PR and TR (thyroid hormone receptor) (4, 5). As an important component of the ubiquitin-proteasome pathway, E6-AP is also assumed to be required for the degradation of the steroid receptors. On the other hand, in vitro studies indicated that E6-

AP acts as a coactivator for the steroid receptors, such as ER, PR, and AR (3). Therefore, it is of great interest to investigate if the expression pattern of this dual function protein is changed in carcinomas of breast and prostate, and what the underlining mechanism is.

Materials and Methods

Human tumor samples. Formalin fixed, paraffin embedded samples of 13 invasive breast carcinomas (IBC) from Baylor College of Medicine Breast Center, 20 ductal in situ carcinoma (DCIS) of breast from Creighton University Medical Center, and 7 prostate carcinomas from the Scott Department of Urology, Baylor College of Medicine were included in this study. The tumor specimens with either internal normal tissue within the same sections (IBC and DCIS) or normal tissues taken from the tumor-adjacent normal area of the same patients (prostate cancer) were sectioned and mounted on the same slide.

Mouse mammary tissues. The inguinal mammary glands taken from E6-AP-knock-out mice, generated in Baylor College of Medicine (6), and wild-type control mice were fixed in 4% paraformaldehyde for 18-24 hrs, embedded in paraffin and sectioned on a microtome.

Immunohistochemistry. Five-μm thick tissue sections were used for immunohistochemistry. After dewaxing and rehydrating, antigen retrieval was achieved by boiling the slides in 0.01M citrate buffer (pH8.0) in a microwave for 6 min.

Endogenous peroxidase activity was blocked by a 30 min incubation of the sections in 1% hydrogen peroxide in methanol at room temperature. This was followed by an overnight incubation with 10% normal goat serum in Tris-buffered saline (TBS) at 4 ^{0}C that blocks the nonspecific binding. The primary antibodies, E6-AP (a rabbit polyclonal antibody, kindly donated by Dr. Norman J. Maitland) and ERα (NCL-ER-6F11, a mouse monoclonal antibody, Novocastra, UK) were used at a 1:200 and 1:100 dilution, respectively, while a dilution of 1:500 for the secondary antibodies, biotinylated goat anti-rabbit for E6-AP and biotinylated goat anti-mouse for ERα, were used. The antibody reactions were carried out at RT for 1 to 2 hrs in a humid condition. The sections were then incubated for 30 min at RT with streptavidin-conjugated peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), and the antibody was visualized with immidazole-DAB (Vector Laboratories, Burlingame, CA) producing a brown colored stain. Finally, the sections were counterstained with hematoxylin, dehydrated through graded alcohol, cleared in xylene and coverslipped for bright field microscopy. Negative controls were included in each experiment by omission of the primary antibody.

Evaluation of immunostained slides. The staining results of E6-AP were evaluated using automated cellular imaging system (ACIS, Chroma Vision Medical systems, Inc., San Juan Capistrano, CA). This system combines color based imaging technology with automated microscopy to provide quantitative information on intensity of staining. Since the expression of E6-AP is ubiquitous in breast or prostate epithelial cells, we compared the intensity of E6-AP staining between normal and neoplastic cells. Nuclear staining for

ERα and AR was evaluated both in terms of intensity and the percentage of positive cells.

***In Vitro* Expression of ER.** *In vitro* expression of radiolabeled human ERα was performed using TNT-coupled rabbit reticulocyte extracts in the presence of [^{35}S]methione according to the manufacturer's recommended condition (Promega, Madison, WI).

Protein Degradation and Ubiquitination Assay. The ^{35}S-labeled human ER α was incubated either with or without E6-AP purified from *E. coli* and in the presence or absence of the proteasome inhibitor, MG132, in a mixture containing 20 mM Tris-HCl (pH7.5), 50mM NaCl, 4mM ATP, 10mM $MgCl_2$, 0.2 mM dithiothreitol, and 4μg of ubiquitin (Sigma) for 1 h at 30^{0}C. Reactions were terminated by boiling samples in the presence of SDS-loading buffer (100 mM Tris-HCl (pH8.0), 200mM dithiothreitol, 4% SDS, 20% glycerol, and 0.2% bromophenol blue). The reaction mixtures were resolved by 10% SDS-PAGE and radiolabeled bands were visualized by autoradiography.

Statistical Methods. Differences of expression between tumor samples and their matched normal tissues were tested using the *Student paired t-test*, while differences of ER expression between IBC and DCIS were tested using *student t-test* of two independent groups.

Results and Discussion

To investigate the expression of E6-AP in human breast cancer, we compared immunohistochemically the expression levels of E6-AP protein in neoplastic cells of IBC and DCIS to the adjacent normal gland. As shown in Figure 1A, in normal breast tissue, E6-AP is strongly and ubiquitously expressed in the cytoplasm of epithelial cells, predominantly in the luminal cells. A weak expression was also noticed in the basal myo-epithelial cells and stromal cells. Neoplastic cells in IBC showed decreased intensity of expression of E6-AP compared to surrounding normal glands (control). All of the 13 IBC samples exhibited decreased immunostaining of E6-AP compared with their normal controls (Figure 1B). On average, there was a 25% reduction of intensity of the immunostaining in tumors than in the normal tissues. Student paired *t-test* indicated that the differences were statistically significant (p=0.00001). However, in DCIS, which represents an intermediate precursor to IBC, decreased expression of E6-AP was not seen (Figure 1A), suggesting that the down-regulation is a relative late event associated with invasive phenotype.

Next, we analyzed the expression of ER α, one of the two estrogen receptors, in the IBC and DCIS samples and compared its expression pattern with that of E6-AP. In IBC, the down-regulation of E6-AP was accompanied by the increased expression of ERα, in the epithelial cells; while in DCIS, high level expression of E6-AP was accompanied by relative low expression of ERα (Figure 2A). Taking the expression of E6-AP in normal tissues as 100%, the relative expression of E6-AP is 106% in DCIS and 75% in IBC (Figure 2B). Furthermore, comparison of the H scores (intensity multiply by percentage) of ERα in IBC and DCIS indicated that there was higher expression of ER α in IBC than

in DCIS (student *t-test*, p=0.001133) (Figure 2C). These data suggested an inverse correlation between the expression of E6-AP and ERα.

It is known that the normal development and function of mammary gland largely depend on the normal expression and function of ER and PR (7, 8). ERα is also an important mitogenic factor for breast epithelial cells. In addition, ER and PR also appear to play significant roles in the development, progression, treatment and outcome of breast cancer (1). The finding of the decreased expression of E6-AP and increased expression of ERα in IBC implied that E6-AP might be a factor that regulates the expression and function of ERα in mammary gland and contribute to its tumorigenesis. Considering the fact that E6-AP is an E3 ubiquitin-protein ligase enzyme, it is possible that E6-AP promotes ER degradation via the ubiquitin-proteasome pathway. If so, loss of E6-AP function may provide growth advantage to the tumor cells.

The ubiquitin-proteasome pathway accounts for the selective degradation of short-lived regulatory proteins including many nuclear receptors. Three classes of enzymes are involved in protein ubiquitination, i.e. the E1 ubiquitin-activating enzyme (UBA), E2 ubiquitin-conjugating enzymes (UBCs) and E3 ubiquitin-protein ligases. The UBA activates ubiquitin and transfers it to one of the several UBCs. Then the ubiquitin is transferred from E2 to the target protein, either directly or through an E3 ubiquitin-protein ligase intermediate (9, 10). UBA and UBCs are necessary for the degradation of steroid receptors including ER (4). Ubiquitin-proteasome pathway is required for the degradation and turnover of ER (4, 5, 11), which permits continuous responses to changes in the concentration of estrogen. As a rate-limiting factor in the ubiquitin-proteasome pathway, it is possible that E6-AP is also required for the turnover and

activity of the receptor proteins. To test this hypothesis, we performed *in vitro* protein degradation and ubiquitination assay. As mentioned in the *Materials and Methods*, ^{35}S-labeled ERα protein was synthesized in vitro by using TNT-coupled rabbit reticulocyte extracts in the presence of radiolabeled methionine. The ^{35}S-labeled ERα protein was then incubated with ATP and ubiquitin either in the presence or absence of bacterially expressed E6-AP. As expected, E6-AP promoted the degradation of ERα (Fig. 3A), and the proteasome inhibitor, lactacystin, could inhibit the degradation. To further confirm that E6-AP modulates the levels of ERα, we also examined the ERα protein levels in E6-AP knockout mice. E6-AP knockout mice exhibited an increased expression of ERα in the mammary epithelial cells (Fig. 3B). These results indicated that ERα is degraded through the ubiquitin-proteasome pathway and E6-AP is essential for ERα degradation.

E6-AP has been identified as a coactivator for ER, PR, AR and GR. By transient transfection assay, it was shown that E6-AP could potentiate the ligand-dependent transcriptional activity of these steroid receptors (3). Recently, the roles of coactivators in tumor development and progression have gained more and more attention. In fact, overexpression of nuclear receptor coactivators, including AIB1 (amplified in breast cancer 1), SRA (steroid receptor RNA activator), and AIB3 (amplified in breast cancer 3), have been implicated in human breast cancers (12-17). It is considered that overexpression of coactivators would upregulate the signaling of steroid receptors, such as ER and PR, which occurs in breast cancer development and progression. As a coactivator for ER and PR, the alteration of E6-AP expression is also speculated in breast cancers. Previously, it was reported that E6-AP is overexpressed in a spontaneous mouse

mammary tumor model, which is ER-negative (18). Taken together, these data indicated that E6-AP is a unique steroid receptor coactivator characterized by its ubiquitin-protein ligase activity. Further study on the expression of E6-AP in different stages of breast cancers and genetic manipulation of E6-AP gene, either mutation of its ubiquitin-ligase activity or the ablation of its coactivation activity, in cells or in animals will help elucidate the exact roles that E6-AP plays in human breast cancers.

Prostate cancer has many similarities to breast cancer in regard to its AR expression, the hormone responsiveness at the earlier stage and the hormone resistance at the later stage (2). Changes of the expression of some AR coactivators have also been reported (19-22). Therefore, we were interested in knowing whether the expression of E6-AP is also altered in prostate cancers. Similar to the result seen in breast cancer, the expression of E6-AP was also decreased in prostate cancers compared with the matched normal tissues from the same patients. In normal prostate tissues, the expression of E6-AP was mostly localized to the cytoplasm of the luminal epithelial cells, while it showed more diffuse expression in tumors (Fig. 4A). Again, all of the 7 tumor samples exhibited a decreased immunostaning of E6-AP compared with their normal tissue controls (Fig. 4B). There was an average of 27% reduction in tumors compared with the normal controls. Student paired *t-test* indicated the differences are statistically significant (p=0.000022). An inverse correlation between the expressions of E6-AP with that of AR was also seen in prostate cancers (data not shown). Besides, the E6-AP-knockout animals also exhibited increased expression of AR (data not shown). These results indicated that E6-AP might

not only participate in the carcinogenesis of breast cancers, but also in the carcinogenesis of other tissues, such as the prostate.

In conclusion, we presented data herein showing that E6-AP is down-regulated in breast and prostate tumors and the expression of E6-AP is inversely associated with that of ER and AR. The roles that E6-AP plays in the carcinogenesis, tumor progression and hormone resistance of breast or prostate probably involve its participation in the degradation of ER or AR through ubiquitin-proteasome pathway.

Acknowledgements

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Figure Legends

Figure 1. The expression of E6-AP is decreased in human invasive breast carcinomas, but not in the ductal carcinoma in situ. A. Immunohistochemistry of human breast tissues for E6-AP expression: a. Nor, normal area; b. IBC, invasive breast carcinoma; c. DCIS, ductal carcinoma in situ. Enlarged images were shown on the right panel, respectively. The formalin-fixed, paraffin-embedded human breast tissue sections were deparaffinized, rehydrated, and subjected to immunohistochemistry study with anti-E6-AP antibody (1:200 dilution, provided by Dr. Norman J. Maitland) as described in *Materials and*

Methods. B. The immunostaining intensity of E6-AP in both the normal areas and IBC areas of each patient, analyzed by automated cellular imaging system (ACIS, Chroma Vision Medical systems, Inc., San Juan Capistrano, CA).

Figure 2. E6-AP is inversely associated with ERα. Immunohistochemistry of human breast tissues for the expression of E6-AP and ERα. A. Comparison of the expression of ERα and E6-AP in IBC and DCIS. a. ERα in DCIS; b. ERα in IBC; c. E6-AP in DCIS; d. E6-AP in IBC. B. Relative expression (shown as intensity) of E6-AP in DCIS and IBC compared with that in their adjacent normal controls. C. Comparison of the the expression of ERa (shown as H scores) of ERa in IBC and DCIS. The formalin-fixed, paraffin-embedded human breast tissue sections were deparaffinized, rehydrated, and subjected to immunohistochemistry study with anti-E6-AP antibody (1:200 dilution, provided by Dr. Norman J. Maitland) or anti-ERα antibody (1:100 dilution, NCL-ER-6F11, Novocastra) as described in *Materials and Methods.* The stained slides were analyzed by automated cellular imaging system (ACIS, Chroma Vision Medical systems, Inc., San Juan Capistrano, CA).

Figure 3. E6-AP promotes the degradation of ERα through the ubiquitin-proteasome pathway. A. Protein Degradation and Ubiquitination Assay: a. Input; b. –E6-AP; c. +E6-AP; d. +E6-AP and lactacystin. Radiolabeled human ERα was synthesized using TNT-coupled rabbit reticulocyte extracts in the presence of [^{35}S]methione. The ^{35}S-labeled human ERα was then incubated either with or without E6-AP and in the presence or absence of the proteasome inhibitor, MG132, in a condition necessary for protein

ubiquitinatina and degradation. Reactions were terminated after 1 hr by boiling samples in the presence of SDS-loading buffer. The reaction mixtures were resolved by 10% SDS-PAGE and radiolabeled bands were visualized by autoradiography. B. ERa expression increases in the mammary epithelial cells of E6-AP knockout mice. a. WT, wild-type mouse mammary gland; b. KO, knockout mouse mammary gland.

Figure 4. The expression of E6-AP is decreased in human prostate cancers A. Immunohistochemistry of human breast tissues for E6-AP expression: a. normal, normal area; b. tumor, tumor area. Enlarged images were shown on the right panel. The formalin-fixed, paraffin-embedded human prostate tissue sections were deparaffinized, rehydrated, and subjected to immunohistochemistry study with anti-E6-AP antibody (1:200 dilution, provided by Dr. Norman J. Maitland) as described in Materials and Methods. B. The immunostaining intensity of E6-AP in both the normal areas and tumor areas of each patient, analyzed by automated cellular imaging system (ACIS, Chroma Vision Medical systems, Inc., San Juan Capistrano, CA).

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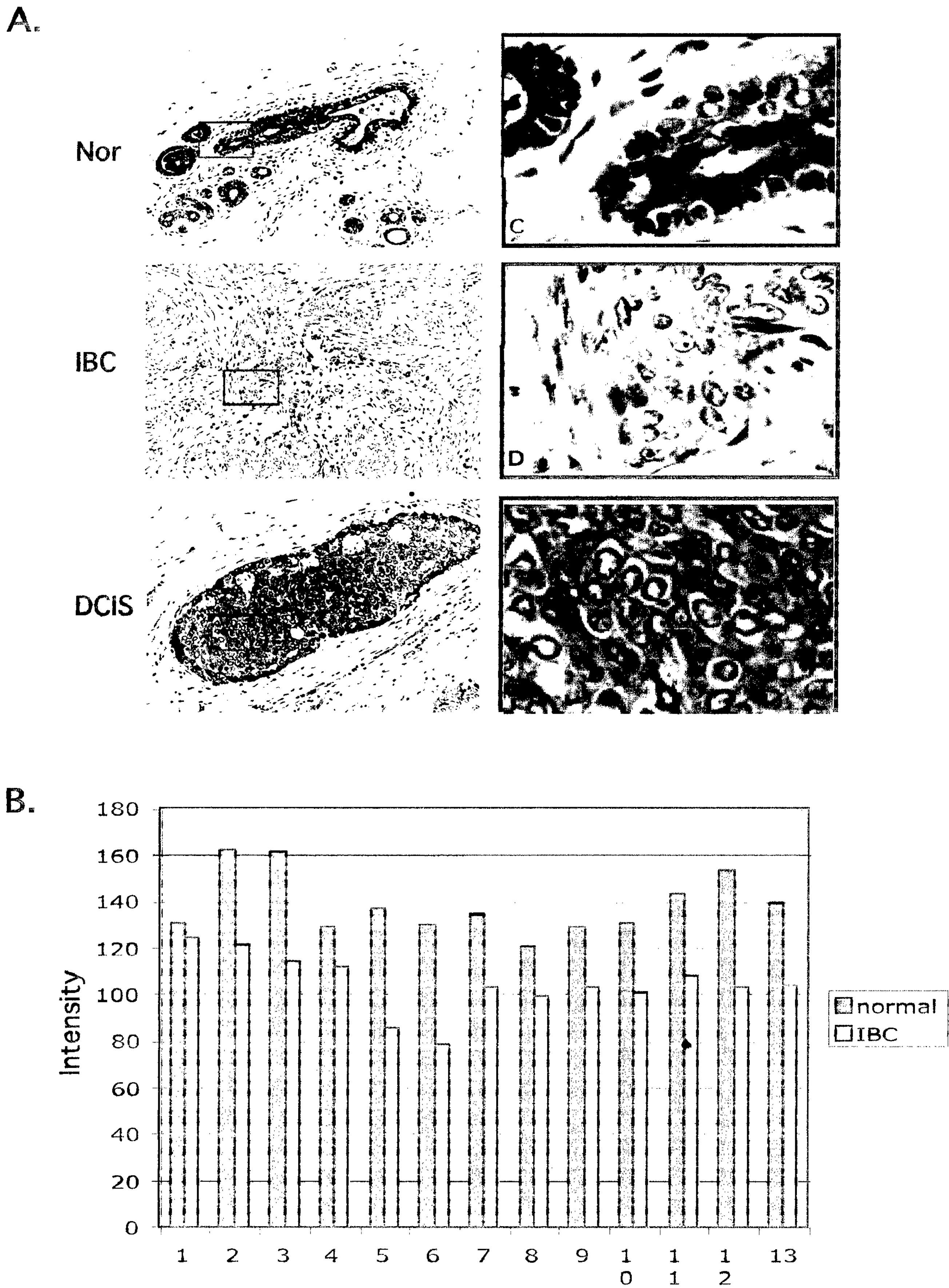
A.
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Figure 1

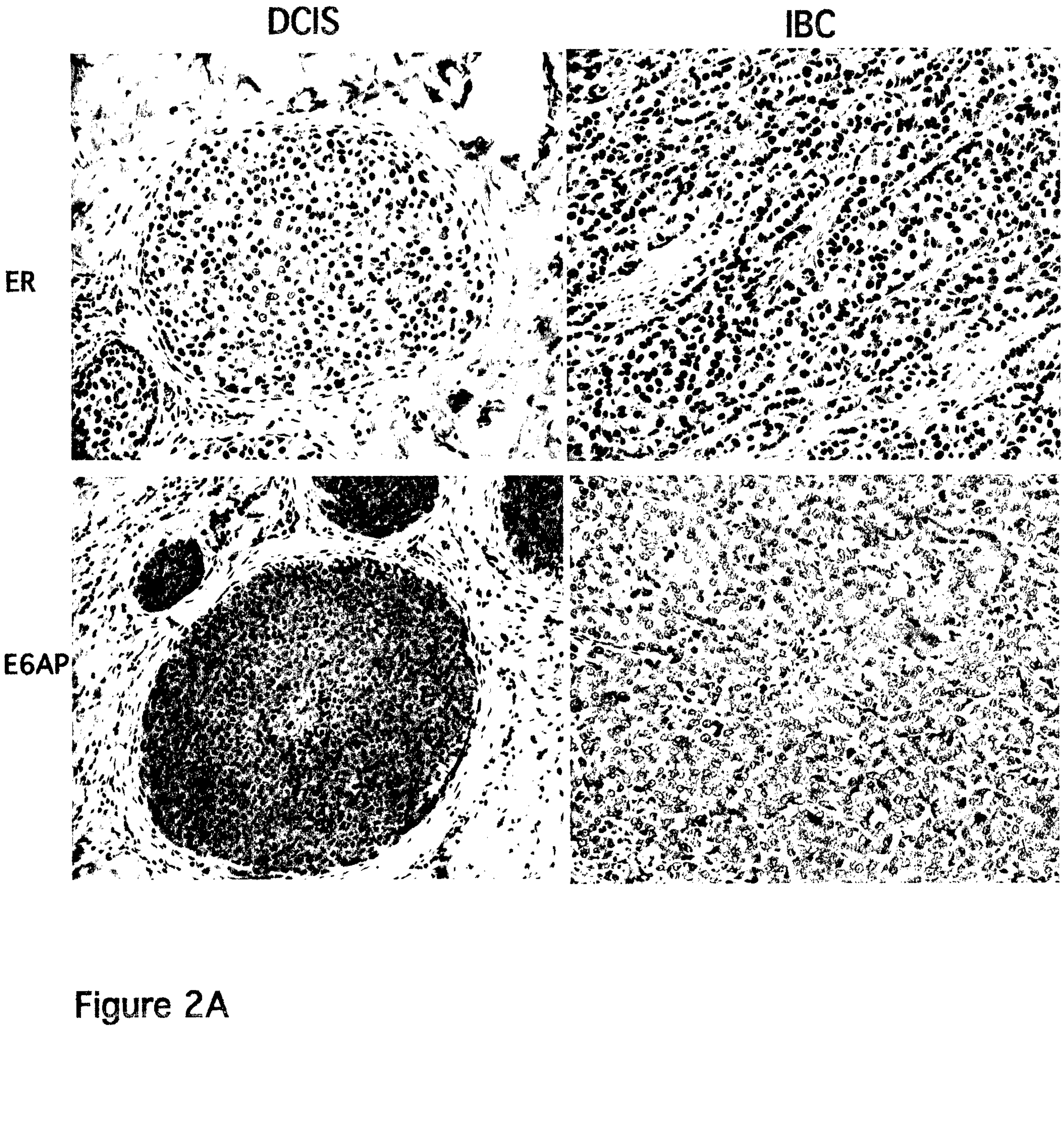


Figure 2A

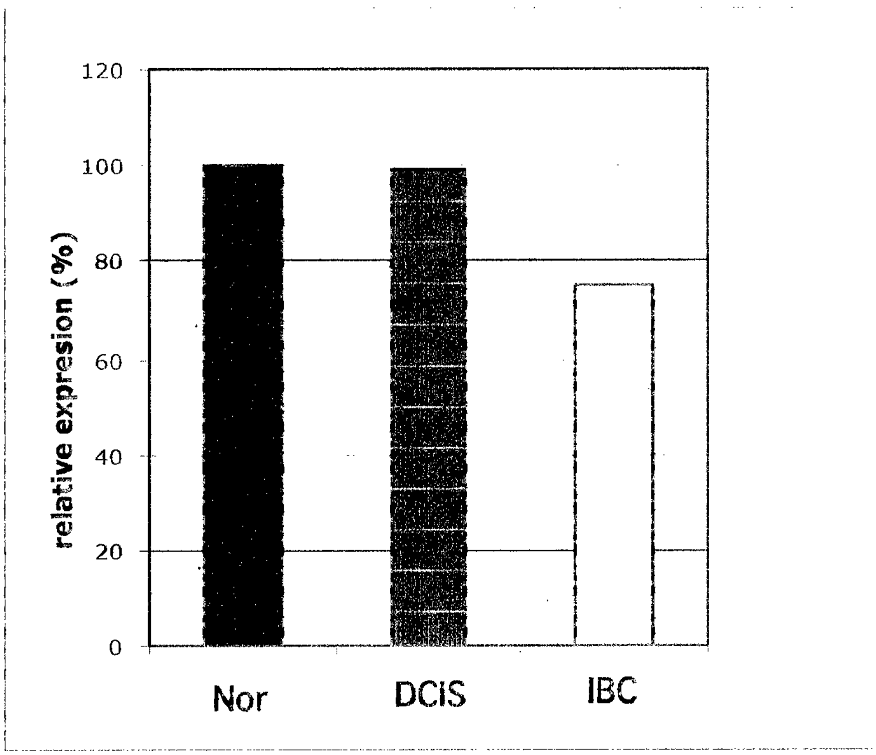


Figure 2B. Relative expression of E6-AP in DCIS and IBC compared with their normal tissue controls.

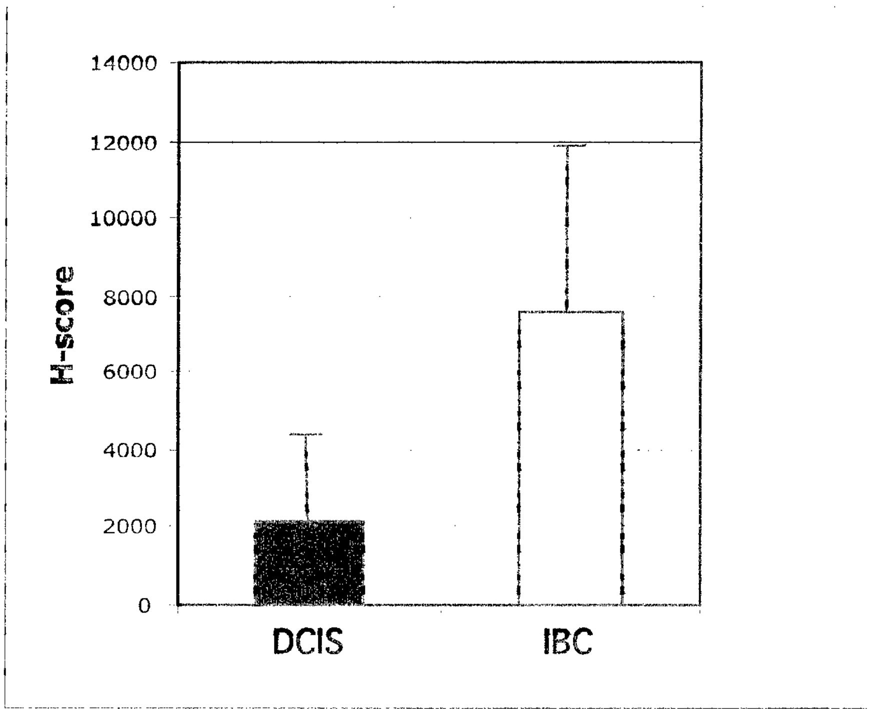


Figure 2C. Expression levels of ERa in DCIS and IBC

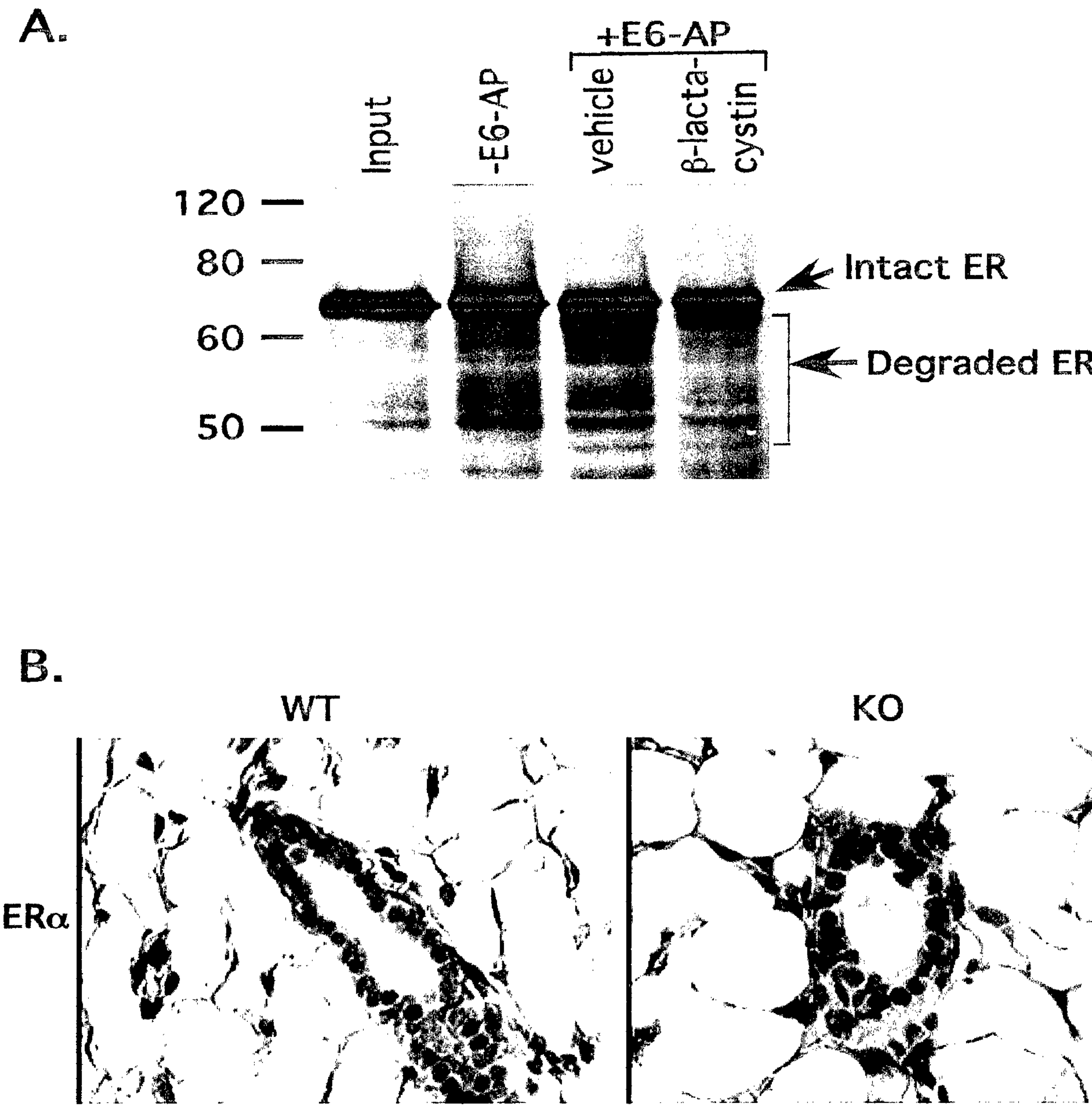
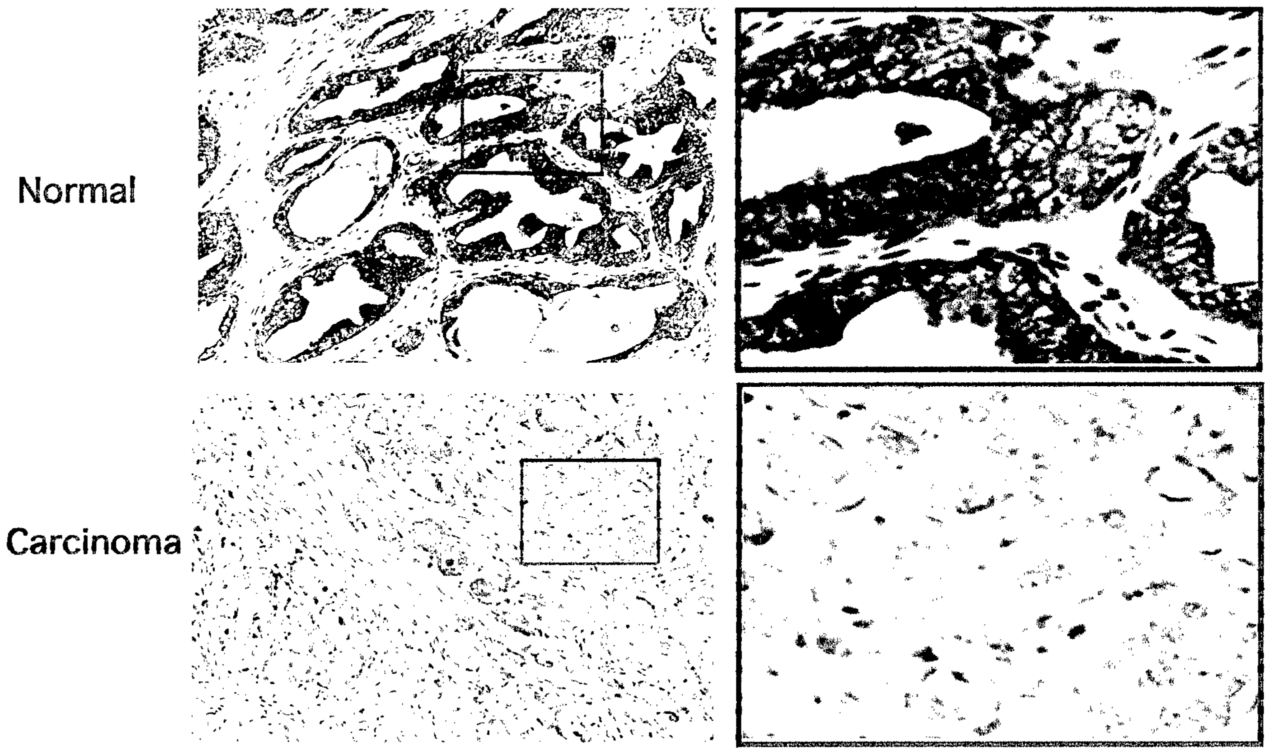

A.
+E6-AP
Input
-E6-AP
vehicle
β-lacta-cystin
120
80
60
50
Intact ER
Degraded ER
B.
WT
KO
ERα

Figure 3

A.


Normal
Carcinoma

B.

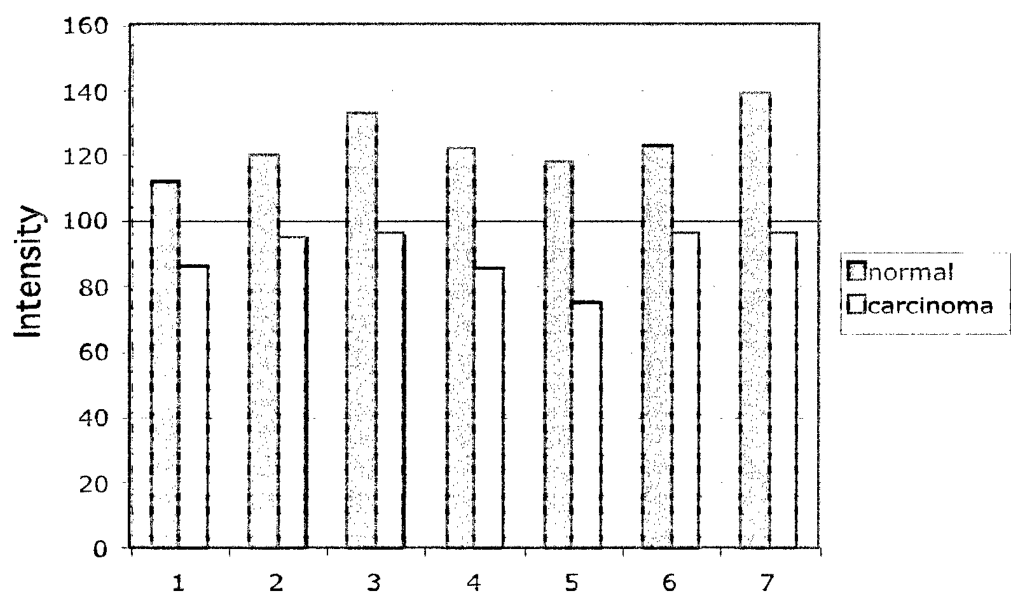

160
140
120
100
80
60
40
20
0
Intensity
1
2
3
4
5
6
7
normal
carcinoma

Figure 4